INVESTIGATING TOLL-LIKE RECEPTOR AGONISTS AS AN IMMUNOTHERAPEUTIC FOR METASTATIC OSTEOSARCOMA

BY

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DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in VMS-Pathobiology
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2018

Urbana, Illinois

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ABSTRACT

This dissertation and the accompanying research is the work of a study designed to determine the plausibility of toll-like receptor agonists as an immunotherapeutic for canine osteosarcoma. From a comparative and translational perspective, the long-term goals of this research include not only the treatment of canine osteosarcoma, but also human osteosarcoma, as this disease has numerous similarities between clinical presentation and progression in both dogs and people. While the translation of our findings into people is far ahead in the future, this understanding facilitated our study approach and helped shape it into not only a systematic research approach, but also an integrated methodology designed to enhance the likelihood of positive outcomes in dogs and possibly even people.

Chapter I entails a review of the past and current literature regarding immune interactions in canine osteosarcoma, as well as immunotherapeutic strategies investigated for the treatment of this canine cancer. Here we also briefly detail the clinical presentation and progression of canine osteosarcoma, along with its similarities to human osteosarcoma. The material reviewed in this section create a solid foundation of the current knowledge surrounding canine osteosarcoma and the immune system, including the substantial finding that osteosarcoma is an immunogenic tumor which can be eliminated by the immune system.

Chapter II describes the *in vitro* studies investigating the dichotomy of toll-like receptor expression and functionality between immune and osteosarcoma cell lines of both murine and canine origin. The aims of this study were to identify several toll-like receptor agonists that would maximize an anti-tumor immune response, but minimally stimulate osteosarcoma tumorigenesis. By investigating mRNA expression levels of a diverse repertoire of toll-like receptors and measuring the response of both immune and osteosarcoma cells to treatment with various toll-like

receptor agonists, which included read-outs such as cellular viability, cytokine secretion, and pro-tumorigenic protein upregulation, we were able to identify 3 toll-like receptor agonists (Pam3CSK4, Poly(I:C), and CpG ODN 2395) that fit our requirements for immune stimulation while minimizing osteosarcoma tumorigenesis.

Chapter III investigates the ability of these 3 agonists to reduce osteosarcoma metastatic lung tumor burden in a clinically-relevant murine model of osteosarcoma, utilizing the highly aggressive K7M2 osteosarcoma cell line. This model employs tail-vein injection of the neoplastic K7M2 cells, which subsequently shower the lungs and consistently replicate the spontaneous occurrence of lung metastasis that is deadly in both dogs and people with osteosarcoma. Here we show that the TLR9 agonist CpG ODN 2395 is highly effective at reducing osteosarcoma lung metastasis in this model and have also detailed the limitations of this therapy. In addition, we investigate a few of the mechanisms that may underlie CpG ODN 2395's efficacy.

Chapter IV explores the safety of single dose CpG ODN 2395, along with its ability to stimulate a measurable immune response in dogs with spontaneous OS in the form of a dose-escalation pilot study. Through measurement of circulating cytokine levels and evaluation of hematologic parameters, we conclude that a single dose of 2 mg CpG ODN 2395 does stimulate an inflammatory response that culminates with evidence of T cell activation, supporting that CpG ODN 2395 is immunologically active in osteosarcoma-bearing dogs. We also report a lack of observable toxicities, making CpG ODN 2395 a strong candidate for future clinical trial exploration in dogs with spontaneous osteosarcoma. **Chapter V** subsequently concludes the dissertation and discusses future directions in regard to further evaluation of CpG ODN 2395 as a possible osteosarcoma immunotherapeutic.

To Gavin, for your unconditional love.

You will never be "just a dog" to me.

And to all the loved pets who have cancer,

someone, somewhere is working hard to save you.

ACKNOWLEDGEMENTS

I am deeply indebted to many individuals for their guidance and support through this process called a "PhD." Without you, I would not be who I am or where I am today…and that deserves more thanks than I can ever give.

Thank you to Morris Animal Foundation, who supported this research with a generous amount of grant funding. We may actually have something, and you made it possible from a monetary perspective (a very big deal!). And to Tim, my advisor, for taking a chance on me, teaching me, and giving me opportunities to use my training as a pathologist. You pushed me to think outside my comfort zone and were the only one to truly believe in my worth as an academic when times were tough. For this last reason, I will be forever grateful. I also want to thank all of my committee members, both past and present, for their mentorship and support. Being on a committee is often thank-less, and I want to emphasize the importance of your role in this process.

Holly Pondenis, Bahaa Fadl-Alla, Renee Walker, Barbara Pilas, David Bunick, Gabriela Calzada-Nova, and Federico Zuckermann all deserve numerous rounds of applause for their technical support and expertise, as some of it was absolutely critical to the scientific integrity and success of this research. I appreciate your willingness to help and to teach with enthusiasm, which is surprisingly hard to find in academia. I have made a mental note to pass that enthusiasm along during my career.

Thank you to Anne Barger and Amy MacNeill. You both knew the benefits of a PhD, even if intangible, and were kind enough to share this knowledge and encourage the pursuit of one. Thanks also to Rick Fredrickson for paying me a bit more to do what I love and believing in the value of an advanced degree. Even though you say nobody likes you, I do.

To everyone at the University of Illinois College of Veterinary Medicine who ever lent me a kind ear when things weren't quite going right, which was often…thank you, thank you, thank you! You all know who you are but shall remain anonymous for posterity.

To my mom, Ruth Anne. Thank you for being a selfless mother. I know you gave up many things so I could have a bright future and a self-supportive career. While some will say that's just what good parents do, there are many "good" parents that would never have sacrificed what you did for me. I only hope I can be half the parent you are, if I ever get this degree done and move on to other things, like having a family.

To Denny, my father. Blood doesn't make a father and you are the epitome of that statement. Thank you for coming into my life and never leaving. Thanks also for all the mad sports skills you taught me (along with Mom, of course) and for teasing me relentlessly the majority of my young adult life. All that teasing taught me to be lighthearted, which has helped me tremendously in the real world.

And finally, to my husband, Andy. You are my rock. You are my best friend. You are my love. I can't wait to spend the rest of our lives together – a new chapter awaits us.

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CHAPTER I

LITERATURE REVIEW

This chapter was accepted for publication in 2015 as a review article by the Journal of Veterinary Internal Medicine.[a]

Abstract

Osteosarcoma is a malignant mesenchymal neoplasm that accounts for the majority of primary bone tumors in dogs and shares biological and clinical similarities with osteosarcoma in humans. Despite dose intensification with conventional cytotoxic therapies, survival times for dogs and humans diagnosed with high-grade osteosarcoma have not changed in the past 20 years, with the principal cause of mortality being the development of pulmonary metastases. Given the therapeutic plateau reached for delaying metastatic progression with cytotoxic agents, exploration of alterative adjuvant therapies for improving management of osteosarcoma micrometastases is clinically justified. Evidence suggests that osteosarcoma is an immunogenic tumor, and development of immunotherapies for the treatment of microscopic lung metastases might improve long-term outcomes.

In this review, the history and foundational knowledge of immune interactions to canine osteosarcoma are high-lighted. In parallel, immunotherapeutic strategies that have been explored for the treatment of canine osteosarcoma are summarized. With a greater understanding and awareness for how the immune system might be redirected toward combating osteosarcoma

[a] Wycislo KL, Fan TM. The immunotherapy of canine osteosarcoma: a historical and systemic review. J Vet Intern Med 2015;29:759-769.

metastases, the rational development of diverse immune strategies for managing osteosarcoma holds substantial promise for transforming the therapeutic landscape and improving disease management in both dogs and human beings.

Introduction

Dogs are second only to humans in terms of naturally occurring inherited disease, and dogs retain breed homogeneity that often mimics certain human demographics such as race or geographic phenotypes.[1] Dogs also acquire similar genetic diseases and cancers as do people and consequently might serve as suitable comparative models for conserved pathologies.[1,2] The accelerated aging of dogs, especially large breeds in comparison with humans, combined with the large numbers of veterinary healthcare dollars spent on dogs (second only to human healthcare dollars spent), provide researchers with a relatively large population of pet dogs that might be available for the study of cancer pathogenesis, as well as for participation in clinical cancer trials.[3,4] Collectively, the shared genetics of specific canine cancers with their human counterparts and the high societal value placed upon dogs as companion animals allow pet dogs to serve as valuable large animal models for translational cancer research.

Osteosarcoma (OS) accounts for 85% of all skeletal tumors in the dog, making it the most common primary bone tumor[5–9] with an estimated 10,000 dogs diagnosed with OS each year.[10] Histologically, OS is composed of malignant mesenchymal cells of stem cell or osteoblast lineage that produce osteoid. Different histologic subtypes of OS exist including osteoblastic, fibroblastic, chondroblastic, and telangiectactic phenotypes, and are based upon the morphology and differentiation characteristics of tumor cells.[11] Typically, OS is considered a disease of large and giant breed dogs[12] and it has a predilection to arise from the appendicular skeleton.[13,14] Middle-

aged to older dogs (median age of 7 years) are most commonly affected by OS,[5–9,13,15–22] but a bimodal age distribution may occur with a smaller peak of OS at 18–24 months of age.[18]

Similar to dogs, OS is the most common primary focal skeletal tumor in people and accounts for more than 56% of all bone tumors. In adolescents, OS is the third most frequent cause of cancer and often affects taller adolescents, similar to large breed dogs.[2] The diagnosis of OS in people also follows a bimodal age distribution, but unlike dogs where the incidence of OS is highest in older animals, adolescents are affected more frequently in humans.[23]

The biologic behavior of OS is aggressive, initially restricted to the local bone microenvironment but with distant organ involvement as a result of metastatic disease progression. Although only about 15% of dogs and 20% of people present with detectable lung metastases, the eventual development of distant metastatic foci in the absence of chemotherapy is 90% within 1 year for dogs and 80% within 2 years for people.[7,10,20,24] Although adjuvant chemotherapy has increased the cure rate of people and survival time of dogs diagnosed with high-grade OS, there has been no improvement in long-term treatment outcomes in the last 20 years for either species, despite the institution of conventional dose intensification strategies.[25–28] Based upon the current therapeutic ceiling reached, the identification of promising and complementary adjuvant treatments for improving the management of micrometastatic disease is clinically warranted.

Focused scientific development and clinical assessment of novel immunotherapeutic strategies are areas that are rapidly gaining momentum in veterinary medicine and in the treatment of canine OS (**Figure 1.1**). Given the conserved biology of OS between dogs and people, unique opportunities exist for veterinary researchers and clinical oncologists to adapt immunobiological advances in the human oncology arena for translational purposes in dogs with OS. Reciprocally, novel tumor immunotherapeutic strategies first evaluated in dogs with OS also have the potential

to inform and guide development of new treatment regimens for the benefit of human cancer patients.

Clinical Evidence of Immunogenicity: Limb-Spare Infections in Osteosarcoma

The capacity for the immune system to recognize and eliminate cancer has been recognized for over a century with some of the earliest reports including the eradication of bone sarcomas. In the early 1890's, William Coley reported that accidental acquisition or intentional inoculation of the bacterium responsible for erysipelas (*Streptococcus pyogenes*) could result in regression or delayed recurrence of various cancers.[29,30] These clinical insights eventually led to Coley's development of a vaccine consisting of 2 killed bacteria, *Streptococcus pyogenes* and *Serratia marcescens*.[31] The vaccine was named "Coley's Toxins" and was efficacious in the treatment of a variety of tumor types, including bone sarcomas.[32]

Coley's observations that underscored the potency of the immune system against cancer have been long recognized, but not until recently was direct *in vivo* evidence regarding infection-enhanced anti-tumor immunity for OS revisited and reported to the medical community. Although histologic evidence of non-septic, chronic inflammation in biopsy specimens of canine OS has not been found to be prognostic,[18] spontaneous regression of OS in dogs has been reported[33] and dogs that experience acute bacterial infection secondary to limb-salvage surgery have been found to have increased survival times in several independent studies.[34–37] The initial observation of this finding was reported in a study investigating the use of radiation therapy before cortical allograft limb-sparing surgery in dogs with high-grade appendicular OS. Although radiotherapy before limb-sparing surgery was deemed detrimental for achieving durable fixation of bone allografts, a

significant increase in survival time was noted between dogs whose allograft became infected as compared to dogs with allografts that remained uninfected (11 versus 5 months, respectively).[34]

These initial findings later were corroborated by 2 additional studies that examined the outcome of dogs with OS treated with limb-sparing surgery and adjuvant chemotherapy.[35,36] Dogs with distal radial OS that developed cortical allograft infection were half as likely to die and half as likely to develop metastatic disease as compared to dogs without infection, which resulted in a significant difference in median survival time (MST) of 18 versus 7.6 months, respectively.[35] Similar findings were reported in dogs undergoing either cortical allograft or endoprosthetic limb-sparing surgery.[36] No difference in MST was found between the 2 surgical groups but MST was found to be significantly longer in dogs that experienced construct failure (22.8 versus 10.7 months) or post-operative infection (22.8 versus 9.6 months). Dogs with post-operative infection also were 25 times less likely to die, and median metastasis-free interval (MFI) was increased for dogs with infection (18.5 versus 9.1 months). All dogs with construct failure also had post-operative infections, and irritation secondary to construct failure might have contributed to infection development. Lastly, in a recent retrospective study, when evaluating only dogs that lived for >1 year after histopathologic diagnosis of OS, increased survival time of dogs that developed post-operative limb-spare infections also was identified. Dogs with limb-spare infections had a MST of 6 months beyond 1 year, whereas dogs that underwent limb-sparing surgery but did not acquire infection only achieved a MST of 0.9 months beyond 1 year.[37]

People with OS who were treated by endoprosthetic replacement and experienced post-operative infection also had increased survival time.[38] A later study, however, found no difference in survival time between infected and non-infected patients when matched for type of chemotherapy, histologic response, tumor size and location, and local recurrence.[39] No case–

control studies for comparison have been performed in dogs to date, but the majority of studies do suggest that non-specific immune stimulation secondary to infection prevents the recurrence or delays progression of OS in a clinical setting.

The immune mechanisms that contribute to increased survival secondary to limb-spare infection have not been well-studied, but evidence for innate system involvement on the suppression of OS growth has been derived from a murine model of chronic bacterial osteomyelitis. In this study, osteomyelitis decreased tumor growth and increased survival time in mice when tumors were established after infection, but this effect was abrogated when tumors were established before induction of osteomyelitis.[40] Several different types of infectious agents have been cultured in affected dogs[34–36] and infection-associated inhibition of tumor growth in the murine model of chronic bacterial osteomyelitis was not dependent on the specific infectious agent involved. Increased circulating and splenic inflammatory monocytes as well as increased tumor-associated macrophages (TAM) were observed in infected mice, and depletion of natural killer (NK) cells or monocytes and macrophages was found to reverse the tumor growth inhibition seen with concurrent osteomyelitis. These observations led the authors to conclude that both NK cells and monocytes and macrophages are associated with the innate anti-tumor response elicited by chronic bacterial osteomyelitis, and they speculated that the increase in inflammatory monocytes was associated with repopulation of activated TAM, which were expected to be tumor-inhibitory in this setting rather than tumor-promoting. Furthermore, the finding of increased NK cells might be related to back-and-forth activation between NK cells and monocytes, also contributing to tumor inhibition.[40]

These clinical and pre-clinical studies strongly suggest that OS is an immunogenic neoplasm, and micrometastatic disease potentially can be controlled or eliminated after recognition

by the immune system. Case–control studies in dogs to either confirm or refute these findings[39] and mechanistic studies to characterize the specific immune responses against OS cells elicited by limb-spare infections are lacking.

Humoral Evidence of Immunogenicity

Cell-Mediated Reactivity and Serum Blocking Activity

Cell-mediated reactivity (CMR) and serum blocking activity (SBA) experiments were used to investigate interactions of the immune system with canine OS. Simply defined, CMR refers to inhibition of target cell growth whereas SBA refers to promotion of target cell growth in the presence of serum. Autologous serum from dogs with progressively growing OS exhibited SBA effects in vitro, and decreased CMR was observed when co-cultures of autologous lymphocytes and tumor cells were incubated in the presence of the patient's serum. In the absence of autologous serum, high numbers of lymphocytes could inhibit tumor growth *in vitro*, whereas low lymphocyte numbers conversely stimulated growth. Based upon these observations, humoral factors present in the patient's serum (e.g., blocking antibodies or antigen-antibody complexes) were surmised to prevent tumor destruction by autologous lymphocytes, but other serum-derived factors likely potentiated tumor growth. Whether the SBA was mediated by inhibitory cytokines was not considered in these studies.[41,42]

Extending upon these initial findings, changes in SBA were investigated pre- and post-operatively in dogs with OS that underwent amputation of the tumor-bearing limb. Increased pre-surgical SBA was noted in dogs that eventually developed metastatic disease, and post-surgical SBA increased before development of overt metastatic disease in the majority of dogs (6/8, 75%). For dogs remaining free of metastasis, SBA was unmeasurable.[43] In a complementary study, post-

surgical SBA in dogs with OS given Bacillus Calmette-Guerin (BCG) intradermally also was found to increase in conjunction with radiographic appearance of metastasis.[44] Based upon these findings, SBA was proposed to be of potential value for determining prognosis, but identification of specific factors mediating SBA (e.g., antibodies or inhibitory cytokines) was not determined in these studies.[43]

C1q Binding Levels

The C1q-binding test is used in immunology to evaluate circulating immune complexes (CIC). A single study evaluated serum C1q-binding levels in dogs with OS, and demonstrated that a large percentage of dogs (46/ 56, 82%) had increased C1q-binding at the time of diagnosis. In a subset of dogs (n = 12) in which serial C1q-binding levels were available for quantification, divergent trends in C1q-binding levels were observed based upon disease status. In dogs that survived up to 1 year post-diagnosis (n = 4), the C1q-binding levels where found to have fallen within normal reference ranges after completion of therapy. Conversely, in dogs that experienced local disease recurrence or distant metastases (n = 8), levels of C1q-binding either remained increased throughout the entire study duration or only transiently decreased before increasing again. The CIC identified in these dogs had characteristics consistent with IgG, but this conclusion was made cautiously because anomalous fractionation results were observed in normal control dogs.[45]

TP-1 and TP-3 Antibodies

TP-1 and TP-3 are murine anti-human monoclonal antibodies (mAbs) created using the hybridoma technique by immunization of mice with human OS cells. These 2 distinct mAbs bind

different epitopes of the same unknown antigen and have been shown to be highly sensitive and moderately specific for human OS. Using immunohistochemistry (IHC) on canine tumor tissues, the TP-1 and TP-3 antibodies also were shown to have useful specificity for canine OS, although chondrosarcomas and several carcinomas were cross-reactive with these antibodies. Staining of normal canine tissues with these antibodies was limited.[46] Extending upon the recognition of conserved epitopes in formalin-fixed tissues, additional studies in OS dogs with ^{131}I- or ^{123}I-labeled F(ab′)$_2$ fragments of TP-1 or ^{18}F-labeled TP-3 Fab fragments showed high specificity of these antibodies for primary and metastatic OS lesions using immunoscintigraphy and positron emission tomography (PET) scanning, respectively.[47,48] Immunoscintigraphic evaluation of I-labeled F(ab′)$_2$ fragments of TP-1 also detected multiple metastatic lesions that were not detectable by conventional radiography.[47] Despite the sensitivity and specificity of TP-1 and TP-3 antibodies for conserved OS epitopes, use of these antibodies for diagnostic purposes, either for IHC or molecular imaging, is limited in both human and veterinary medicine, and likely stems from a lack of commercial availability. Nonetheless, cross-reactivity of TP-1 and TP-3 antibodies for conserved OS epitopes in canine tumor tissues further supports the capacity for immune recognition of canine OS antigens.

Negative Cellular and Soluble Regulators: Evidence for Immune Evasion

Regulatory T-cells

Regulatory T-cells (T_{regs}) are a component of the immune system responsible for controlling and suppressing excessive immune activation. Phenotypic characterization of T_{regs} includes concurrent expression of CD4 and CD25 surface antigens along with transcription of the FoxP3 gene. Dysregulation of T_{regs} has been incriminated in the induction of autoimmunity, and

conversely promotion of ineffective anti-tumor immunity. Given the potential role of T_{regs} in suppressing the immune surveillance of cancer, T_{regs} have been investigated in dogs with cancer and specifically in dogs with OS. Several studies have documented increased expression of $CD4^+FoxP3^+$ T_{regs} in dogs with cancer compared to controls, findings that suggest potential participation of T_{reg}-induced immune suppression and cancer progression. When stratified by tumor histology, no difference in the percentage or absolute number of T_{regs} was identified between OS-bearing and normal dogs in either peripheral blood or draining lymph nodes, but the small sample sizes of dogs with OS used for comparison might have limited the power to detect the existence of a true difference between groups.[49,50] A later study examining only dogs diagnosed with OS and free of measurable metastatic disease also confirmed no difference between peripheral blood or lymph node $CD4^+CD25^+FoxP3^+$ T_{reg} numbers when compared to healthy controls. In fact, T_{reg} numbers were found to be significantly lower in the tumor-draining nodes when compared to non-draining nodes of the OS dogs.[51]

Discordant with the findings derived from other investigations, differences in T_{regs} were identified in another study that evaluated dogs with OS that had not received chemotherapy within 3 weeks of blood collection. Significantly increased numbers of relative and absolute circulating $CD4^+FoxP3^+$ T_{regs} were identified in OS dogs versus control dogs. Despite differences in T_{regs} identified in the blood, similar differences between OS-bearing and normal dogs were not identified when T_{regs} were evaluated in draining or non-draining lymph nodes. Additionally, no differences in circulating T_{regs} were noted between pre- and post-amputation blood samples. Concordant changes in effector T lymphocyte populations also were examined, and decreased numbers of circulating $CD8^+$ cells (absolute and relative) as well as a decreased $CD8^+$:T_{reg} ratio were observed in dogs with OS. The effector to regulatory T lymphocyte ratio provided prognostic

information, with a low $CD8^+$:T_{reg} ratio being associated with decreased survival as compared to a high ratio.[52]

Myeloid-Derived Suppressor Cells

Myeloid-derived suppressor cells (MDSCs) are immature cells of myeloid lineage derived from bone marrow progenitor cells. In diseases such as cancer, MDSCs are increased and contribute to a global immunosuppressive state. In veterinary medicine, the identification of MDSCs as a small percentage of circulating white blood cells in normal dogs and tumor-bearing dogs has been possible using flow cytometry. In 1 study, the percentages of circulating putative MDSCs identified by $CD11b^{low}$ and $CADO48A^{low}$ surface expression were quantitatively different between normal healthy dogs and dogs diagnosed with different tumor types including OS.[53] A higher percentage of MDSCs was identified in tumor-bearing dogs (7.9%) compared to normal dogs (3.6%), and *in vitro* generated MDSCs possessed the capacity to suppress concanavalin A-induced splenocyte proliferation. In a complementary study, granulocytic MDSCs were identified by $CD11b^+CD14^-MHCII^-$ surface expression in healthy dogs, dogs with early stage non-metastatic cancers, and dogs with advance stage metastatic cancers.[54] In dogs with advance stage metastatic cancers, which included OS and hemangiosarcoma, the percentage of circulating MDSCs was significantly higher than in healthy dogs and those with early stage non-metastatic cancers. Similarly, isolated MDSCs exhibited immunosuppressive activity as demonstrated by attenuation of concanavalin A and human recombinant IL-2 (hrIL-2)-stimulated T cell proliferation. Based on these 2 investigations, evidence was generated to substantiate the existence of MDSCs in dogs, both in health and disease. Importantly, both studies identified increases in the percentage of circulating MDSCs in dogs diagnosed with highly metastatic tumors such as OS.

Despite their proven existence in dogs with cancer, MDSCs have not been definitively identified to play a role in the progression of canine OS. They have been speculated, however, to exert some form of negative immunomodulation in dogs with OS, because dogs with monocyte counts $>0.4x10^3$ cells/μL have a shorter median disease-free interval (DFI) (6.7 versus 15.5 months). In the majority of dogs evaluated (59/69, 86%) this monocyte count did not represent a monocytosis, and monocyte numbers were still within the reference interval.[55] In a study investigating prognostic factors in dogs with OS of the maxilla, mandible, or calvarium, there was also a significantly increased hazard of death with increasing monocyte count.[56]

Tumor-Derived Soluble Factors

Tumor-derived soluble factors (TDSFs) produced from 2 immortalized canine OS cell lines (OSA8 and OSA16) have been shown to suppress the function of cultured canine myeloid cells. Co-culture of canine dendritic cells, macrophages, or both with tumor-conditioned media (TCM) containing TDSFs suppressed activation of these antigen-presenting cells by decreased expression of MHC Class II and CD80 (B7.1), decreased phagocytic activity, and decreased capability to induce splenic effector cell proliferation.[57]

Immunotherapeutics: Historical and Current Strategies

Bacillus Calmette-Guerin

Although utilized for vaccination against tuberculosis since the 1920's,[58] Bacillus Calmette-Guerin (BCG) immunotherapy also has been investigated since the 1930's as an anti-tumor immune modulator after the observation that people who died from tuberculosis coincidently also had a decreased incidence of cancer. Broad anti-tumor immune activity elicited

by BCG has been demonstrated in several murine tumor model systems, as well as in various naturally occurring tumor types including stomach cancer, melanoma, and leukemia. Today, BCG's principal anti-cancer immunotherapeutic role is for the treatment of non-muscle invasive bladder cancer in people.[59] In the setting of bladder cancer, BCG is believed to exert its immunobiologic effects by upregulation of MHC II molecules in malignant transitional epithelial cells, along with the induction of $CD4^+$ T_h1 and cytotoxic T lymphocyte (CTL) responses.[60]

The exploration of BCG immunotherapy for canine OS began in the early 1970's. Initially, injection of BCG either IV, intraperitoneal, or intrathoracic into normal dogs was performed to investigate the capacity of BCG to create pathologic lesions. Histologically, BCG injections generated granuloma formation within the liver and lung parenchyma, along with lymphoid hyperplasia of the tonsils and bronchial lymph nodes. Positive tuberculin test reactions were noted most often in dogs receiving intrathoracic BCG. Subsequently, dogs with spontaneous OS, the majority of which did not have evidence of metastatic disease, were given IV BCG at variable intervals, with or without concurrent vaccination with irradiated autologous tumor cells. Findings derived from these OS-bearing dogs receiving BCG indicated no enhancement of metastasis, but instead a possible delay in metastatic development and progression, conclusions that were substantiated by longer survival times in dogs that received BCG as compared with a historical control group.[61] Another study reported findings derived only from dogs with OS that underwent amputation (n = 12) and that received an identical BCG IV injection scheme, with or without irradiated autologous tumor cells.[62] This study noted a significant increase in survival compared to a previously published historical control group (51 versus 14 weeks).[62] Interestingly, improvements in survival times were not restricted by the route (IV) of BCG administration, and

intradermal delivery of BCG to dogs with OS after amputation also significantly extended survival to 40 weeks versus 13 weeks for controls.[44]

After documented clinical activity in dogs with OS, mechanistic studies were conducted to characterize the potential mode of action of BCG. Studies conducted in normal and tumor-bearing dogs demonstrated that BCG administration could stimulate alveolar macrophage activity and promote lymphocyte cytotoxicity *ex vivo*. Not surprisingly, the anti-tumor cytotoxicity induced by BCG was observed to be non-specific with lysis of several tumor types in addition to OS, including melanoma and mammary carcinomas.[63-65] Non-specific cytotoxicity exerted by BCG-primed alveolar macrophages and lymphocytes against multiple cancer cell lines was suspected to be the result of shared tumor cell antigens or polyclonal lymphocyte activation.[63] Given its capacity to induce granuloma formation, BCG's immunobiologic effects could be associated with activation of circulating monocytes or tissue macrophages in reticuloendothelial organs. Specifically, $CCR2^+$ inflammatory monocytes that expand within the marginal zone of the spleen have been associated with cross-presentation of tumor antigens and subsequent tolerance of $CD8^+$ memory T-cells, whereby splenectomy restores lymphocyte and antitumor function.[66] As such, the effect of splenectomy was evaluated in dogs with OS treated with amputation and administration of an intra-dermal methanol-extracted residue of BCG (MER).[67] Unexpectedly, splenectomy led to decreased survival in the OS dogs, whereas dogs treated with amputation and MER without splenectomy had similar survival times to historical controls.[67]

Muramyl Peptides

The muramyl peptides include muramyl dipeptide phosphatidylethanolamine (MDP), a synthetic analog of the peptidoglycan cell wall of mycobacteria that is the smallest part of

mycobacteria that is immunostimulatory, and muramyl tripeptide phosphatidylethanolamine (MTP-PE), a lipophilic derivative of MDP.[68,69] The liposomal form of MTP (L-MTP-PE) can be used for the *in vivo* stimulation of macrophages and monocytes rendering them cytotoxic against tumor cells, but L-MTP-PE itself does not exert any direct tumor cytotoxic properties.[70,71] These unique features make LMTP-PE an ideal candidate for the immunotherapy of cancer, and in people, L-MTP-PE has resulted in prolonged survival in patients with OS in both initial and relapsed settings.[72-73]

In the context of canine OS, the *in vitro* incubation of canine peripheral blood mononuclear cells (PBMCs) with MDP was shown to elicit cytostasis against the D-17 cell line, and this cytostatic effect was maximized by a combination of MDP and lipopolysaccharide (LPS) compared to either agent used alone.[74] This maximal effect of MDP and LPS was identified to be secondary to TNFα secretion and not because of direct toxicity induced by MDP or the combination of MDP and LPS.[75] Correlating with cell culture experiments, PBMCs collected from healthy dogs after IV L-MTP-PE administration also had significantly more cytostatic activity against the D-17 line, and serum harvested from the same dogs had increased TNFα activity within 2 hours after L-MTP-PE injection.[74-75]

In addition to evaluating the anti-cancer effects of L-MTP-PE as a single agent, a combination of L-MTP-PE and doxorubicin chemotherapy also was investigated *in vitro* and *in vivo*, and indicated that a combination of doxorubicin and L-MTP-PE enhanced PBMC activation and cytotoxicity compared to either drug alone. Again, the observed biologic response was mediated by TNFα.[76] Given the predilection of OS to metastasize to the pulmonary parenchyma, a similar combination platform was investigated for the activation of canine pulmonary alveolar macrophages against D-17 cells. Similar to the results generated by PBMCs, pulmonary alveolar

macrophages harvested from dogs receiving combination therapy with doxorubicin and L-MTP-PE exerted the greatest *ex vivo* cytotoxicity against canine OS target cells.[77]

The translational relevance of the observed in *vitro* and *ex vivo* activity of L-MTP-PE against canine OS cells has been investigated in dogs with spontaneously occurring OS. In the first reported study of L-MTP-PE in dogs with OS, the anti-cancer activity induced by IV L-MTP-PE as a single agent was evaluated by a randomized, placebo-controlled trial. In comparison with placebo controls, dogs treated with amputation and twice weekly IV L-MTP-PE had significant extensions of MST (222 versus 77 days) and median MFI (168 versus 58 days).[78] Despite the substantial improvement in survival times induced by single agent L-MTP-PE, over 50% of the dogs studied ultimately experienced metastatic progression and were dead by 8 months after therapy. In an attempt to improve long-term survival in dogs with OS treated with L-MTP-PE, a subsequent study evaluated the use of L-MTP-PE in combination with adjuvant cisplatin chemotherapy in dogs without macroscopic metastatic disease. When used in combination serially, administration of L-MTP-PE after amputation and cisplatin chemotherapy significantly prolonged MST (14.4 versus 11.2 months) and median DFI (9.8 versus 7.6 months). However, if L-MTP-PE was given concurrently with cisplatin chemotherapy in the adjuvant setting, no survival benefit was identified beyond that achieved with cisplatin chemotherapy alone.[79] Given that L-MTP-PE failed to be approved by the US Food and Drug Administration in 2007 for the initial treatment of non-metastatic OS in children, clinical research with muramyl peptides for the treatment of OS in dogs has not advanced during the past decade, and L-MTP-PE will not likely be available in the United States for the management of OS in dogs. In contrast, L-MTP-PE is approved for use in the European Union for non-metastatic OS in children, as mifamurtide (Mepact®).

Interleukin-2 Cytokine Therapies

Interleukin-2 (IL-2) is a pleiotropic cytokine responsible for several key immune responses including differentiation and subsequent expansion of activated T cells into effector and memory T cells after stimulation by immunogenic antigens. Given the potent role of IL-2 for establishing cell-mediated immune responses, IL-2 has been investigated both *ex vivo* and *in vivo* for the management of canine OS. In an early study, the ability of hrIL-2 to activate peripheral blood leukocytes (PBL) derived from either normal dogs or dogs with lung tumors was investigated *in vitro*. When derived from dogs with lung tumors, autologous stimulated lymphocytes (ASL) generated by culture with hrIL-2 and phytohemagglutinin (PHA) were significantly more cytotoxic against autologous tumor cells when compared with ASL produced from healthy dogs. These findings suggested that hrIL-2 could preferentially reactivate ASL collected from tumor-bearing dogs to exert cytotoxicity against naturally occurring pulmonary tumors, including metastatic OS. In addition, increased ASL cytotoxicity in tumor-bearing dogs compared to healthy dogs could indicate that tumor-bearing dogs had tumor-specific lymphocytes.[80] In a separate study in which healthy beagle dogs were given continuous IV IL-2 via the splenic artery or inferior vena cava, the generation and cytotoxicity of lymphokine-activated killer (LAK) cells derived from PBL against the canine D-17 OS cell line was evaluated. Splenic artery infusion of IL-2 was consistently observed to stimulate LAK cell activity in PBL, whereas inferior cava infusion did not. The spleen also underwent "lymphoblastic change" with splenic artery infusion, which consisted of marked lymphoid proliferation with loss of normal splenic architecture, supporting that the more robust LAK activity seen in these dogs was directly related to splenic immunostimulation.[81] Although

this study evaluated D-17 OS cells as the target cells of LAK activity, it was not further determined if the LAK activity generated was specific for OS or a non-specific tumor response.

Several studies evaluating the immunobiologic activities of IL-2 administration *in vivo* have been conducted and focused on direct delivery of IL-2 or the transgene expression of IL-2 in the lungs of healthy dogs and in dogs with metastatic OS. Nebulization of liposomal hrIL-2 in healthy dogs was shown to activate cytolytic and cytostatic activity of bronchoalveolar lavage (BAL) leukocytes, as well as increased total BAL leukocyte numbers with concurrent increases in the lymphocyte and eosinophil percentages. Additionally, nebulized liposomal hrIL-2 was determined to be safe with no life-threatening adverse systemic reactions noted.[82] When evaluated in a clinical trial of dogs with primary lung tumors or lung metastases, treatment with liposomal hrIL-2 nebulization resulted in 2 of 4 dogs with OS lung metastases experiencing complete regression of all metastatic lesions (lung or lung and lymph node) for >1 year. Interestingly, liposomal hrIL-2 nebulization failed to generate uniform responses in dogs with metastatic OS, with the other 50% of dogs experiencing progressive disease. Correlating with the observed clinical responses, immunobiologic activity of liposomal hrIL-2 nebulization was supported in tumor-bearing dogs by augmentation in BAL leukocyte numbers, percentages, and cytolytic activities. Additionally, an increase in T lymphocytes and a shift in the CD4:CD8 ratio because of increased $CD4^+$ cells was observed in dogs receiving hrIL-2 nebulization. Ultimately, all dogs developed neutralizing antibodies against hrIL-2, limiting application of this immunostimulatory strategy to short-term use in a clinical setting.[83]

In an effort to localize IL-2 preferentially to the lung parenchyma without the need for aerosolization as well as to avoid neutralizing antibody formation, IV administration of cationic liposome-DNA complexes (LDCs) containing canine IL-2 cDNA also has been explored in dogs

with metastatic lung OS (n = 20), with the intent to induce lung-specific IL-2 transgene expression and immunomodulation. Increased NK cell activation, along with increased monocyte expression of MHC Class II and B7.2 (CD86) were observed in this study, indicating the enhancement of immunobiologic activities by LDCs. The LDCs were primarily taken up by $CD11b^+$ monocytes rather than lymphocytes, suggesting that LDCs along with transcription of IL-2 itself both contributed to a systemic, innate immune response. In 3/20 (15%) dogs treated with LDCs, complete or partial regression of lung metastases was observed and 4/20 (20%) dogs experienced disease stabilization. Overall survival time compared to historical controls matched for age and tumor stage also was marginally increased (MST of 2.7 versus 2 months).[84]

Although these 2 in vivo studies exploring IL-2 cytokine strategies identified only modest clinical benefits, the data do provide evidence supporting the feasibility of targeted, lung-specific immunotherapy for the treatment of macroscopic OS pulmonary metastases. Presumably, IL-2 cytokine therapy would be expected to exert greater therapeutic benefit in a microscopic residual disease setting.

Adoptive Transfer of T cells

Only a few studies have investigated the use of adoptive T cell transfer in dogs with OS. One investigation evaluated the feasibility, toxicity, and therapeutic outcome associated with IV infusion of the human cytotoxic T cell line, TALL-104, to dogs with OS. In this study, dogs with appendicular OS were treated with surgery and adjuvant cisplatin chemotherapy, and if remaining free of pulmonary metastases at the completion of chemotherapy, subsequently were given IV infusions of TALL-104 monthly for up to 9 consecutive months. Collectively, combination therapy inclusive of surgery, cisplatin chemotherapy, and adjuvant TALL-104 was tolerable and

allowed OS-bearing dogs to achieve MSTs and DFIs of 11.5 and 9.8 months, respectively, which is comparable to dogs treated with combined surgery and chemotherapy. As expected, the xenogeneic nature of TALL-104 caused dogs to develop antibodies and a cellular immune response against TALL-104 with generation of a long-lived, neutralizing antibody response in some dogs. Mechanistically, anti-cancer activities exerted by TALL-104 were believed to be principally mediated by endogenous anti-tumor immunity and partially by MHC-independent NK cell cytotoxicity, as supported by an *ex vivo* ^{51}Cr-release assay conducted in dogs before relapse.[85]

With unprecedented advances in immunobiological techniques, it recently has become possible to create engineered T cells that express stable chimeric antigen receptors (CARs) with specificity against a myriad of targetable epitopes, thereby allowing for the immunologic recognition and treatment of different types of cancer. Recently, protocols have been optimized to allow for the generation of canine T cells expressing CARs for HER2, a membrane protein expressed in canine OS. Preliminary results indicate that canine T cells with CARs specific for HER2 possess the capacity to kill HER2^{+} canine OS cells in an antigen-dependent manner.[86] Based upon these promising early results, additional studies utilizing molecular CAR technologies for the treatment of dogs with OS are anticipated.

Fas Receptor and Fas Ligand Signaling

Induction of apoptosis is one way the immune system can eliminate cancer cells. Mechanistically, cytotoxic T cells or NK cells can trigger programmed cell death in targeted cells by the coordinated release of perforin and granzyme after appropriate cellular recognition cues. However, all too often, this form of direct apoptosis induction by the immune system can be evaded by cancer cells through their acquisition of genetic mutations. Rather than relying directly on

immune cell induction of apoptosis by cytotoxic T cells, NK activation, or both, investigators have evaluated the ability to induce apoptosis in cancer by indirect mechanisms. One strategy for augmenting immune-mediated apoptosis in cancer cells has been through manipulation of the Fas receptor (Fas) and Fas ligand (FasL) signaling pathway.

Fas-dependent apoptosis is mediated through the binding of FasL, expressed by immune effector cells, with cognate Fas receptor expressed on the surface of target cancer cells. Binding of FasL with the Fas receptor results in clustering of intracytoplasmic death domains and consequent cleavage of initiator procaspases by proximity-mediated activation. In the context of clonal evolution, tumor cells would favor the loss of Fas receptor expression with consequent enrichment of tumor cell populations inherently resistant to Fas-mediated cell death. In support of such a resistance mechanism, lung metastases in people with OS often fail to express the Fas receptor, in contrast with the primary tumors which are Fas^{+}.[87] Additionally, preclinical murine models of OS recapitulate this immune evasive phenomena, with the expression of Fas receptor being lost during OS metastasis, which allows metastatic lesions to circumvent the induction of apoptosis by FasL-expressing effector immune cells, as well as the constitutive cellular expression of FasL within lung tissue.[87,88]

Given the apparent importance of Fas-mediated signaling in the immune surveillance of OS metastases, strategies that modulate the expression of Fas or FasL might augment anti-tumor immune responses. In one study, neoadjuvant FasL gene therapy using an adenovirus vector (Ad-FasL) was delivered to primary bone tumors in dogs with appendicular OS to augment intra-tumoral apoptosis, inflammation, and consequent innate immune responses. In this study, dogs were given a single dose of Ad-FasL, which was followed by a 10-day waiting period after which the dogs underwent amputation of the affected limb. Improved survival was appreciated in dogs

whose tumors had inflammation or lymphocyte-infiltration scores of >1 and in dogs with apoptosis scores (via cleaved caspase-3 IHC) in the upper 50th percentile. Additionally, decreased tumor Fas expression was associated with increased inflammation, DFI, and MST.[89]

Derived from preclinical murine studies indicating that gemcitabine (a deoxycytidine analogue) could cause apoptosis of OS cells through Fas/FasL interactions, a study evaluating the tolerability and anti-cancer activity of aerosol gemcitabine in dogs with OS was conducted. In this study, gemcitabine caused increased apoptosis as measured by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) and marked necrosis within lung metastatic lesions. Metastatic foci of gemcitabine-treated dogs also had increased Fas expression compared to the primary tumor and also when compared to controls, suggesting gemcitabine therapy reverses the down regulation of Fas that is often recognized in metastatic OS lesions. Despite evidence for beneficial immunomodulatory changes induced by gemcitabine aerosolization, little clinical benefit was detected in treated dogs, with median DFI and overall survival time being comparable with historical controls.[90]

Therapeutic Tumor Vaccines

Recently, various tumor vaccine platforms have been investigated as novel treatment strategies for improving the management of diverse tumor types including canine OS. In one study intended to augment innate immune responses generated within the tumor microenvironment, an IV, attenuated *Salmonella typhimurium* (VNP20009) that preferentially localizes and proliferates within tumor tissues, was evaluated in a limited number of dogs with OS (n = 4) and produced modest anti-cancer activities as indicated by a partial response achieved in one dog. Despite some evidence of anti-tumor immune activation, numerous adverse effects also were noted in this study,

limiting the use of VNP20009 to primarily a research setting.[91] In addition to a bacterial agent for enhancing immune responses to OS cells, an oncolytic vaccinia virus (strain LIVP6.1.1) also was successfully tested *in vitro* for its ability to lyse D-17 cells. Although the oncolytic vaccinia virus strategy produced potent localized innate immune responses in murine xenograft models,[92] the translational evaluation of such oncolytic viral strategies has yet to be reported in dogs with OS.

Sophisticated combination cytokine vaccine strategies have been evaluated in dogs with OS and have produced early evidence of activity. In one study, the effects of vaccination with irradiated autologous or allogeneic OS tumor cells with xenogeneic cytokine-producing cells (hGM-CSF and hIL-2) administered subcutaneously plus suicide-gene therapy with ganciclovir (GCV) delivered either intratumorally or peritumorally was evaluated in dogs with appendicular or axial OS (n = 5). To stimulate a robust innate immune response, the combination vaccine strategy incorporated a herpes simplex virus thymidine kinase (HSVtk) suicide gene that had been shown to sensitize transfected cells to GCV. Clinically, the combination cytokine vaccine strategy exerted modest anti-tumor activities, with 1 dog with appendicular OS achieving a partial response, and 2 dogs with axial OS maintaining stable disease. Survival time for the 5 OS dogs ranged from 96 to >386 days, with most dogs living <1 year.[93]

A recent pilot study in healthy beagle dogs investigated the ability of a dual vaccination platform comprised of DNA electroporation and adenovirus serotype 6 (Ad6) for the induction of an immune response against 2 potential targetable tumor-associated antigens, telomerase (TERT), or HER2/neu. Both vaccines were found to induce polyspecific T cell responses, supporting the ability of the vaccines to elicit quantifiable immune activation, and the TERT vaccine was found to significantly increase the number of $CD8^+$ cells. These immune responses were induced with just one injection but could be maintained over time with repeated injections.[94]

With the demonstration that immune responses could be generated in healthy dogs against HER2/Neu, another group has adopted an innovative approach to test if clinically relevant immune responses can be evoked against HER2/Neu-expressing canine OS cells, with a resultant delay in micrometastatic disease progression in dogs with OS. Through the use of a recombinant HER2/neu-expressing *Listeria monocytogenes* vaccine, preliminary results have been promising with increased survival times in dogs receiving *Listeria monocytogenes* vaccination in comparison with historical controls (N. M., personal communication). Although early in its clinical assessment in dogs with micrometastatic OS, the reported findings generated by the investigational *Listeria monocytogenes* vaccine raise exciting possibilities for the future of therapeutic vaccination as a transformative and complementary strategy for improving long-term treatment outcomes in dogs with OS.

Conclusion

A large body of scientific and clinical evidence exists supporting the immunogenicity of canine OS. Given the therapeutic plateau reached with conventional cytotoxic therapies for the management of bone sarcomas in both dogs and people, substantive impetus exists for the focused development and validation of innovative immunotherapeutic platforms for improving long-term disease management. Although new immunotherapeutic platforms potentially could emerge as potent single-agent therapies for canine OS, adjuvant or combination therapies employing both immunotherapy and cytotoxic chemotherapy also could create substantial impact in the therapeutic management of canine OS. Many of the immunotherapies currently investigated have indicated only limited capacity to substantially extend survival time compared to standard treatment or are still in preliminary phases of testing. Nonetheless, continued research in how to best harness the

immune system to combat OS micrometastatic disease remains a highly desirable treatment strategy that holds promise to transform the management of metastatic bone sarcomas in dogs and human beings.

FIGURE

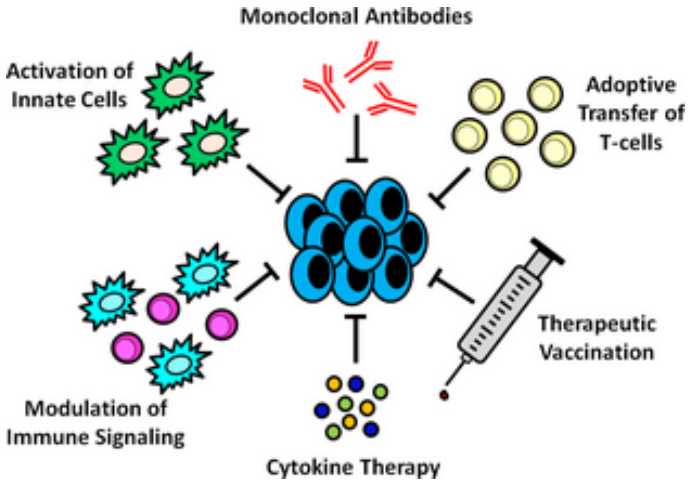

Figure 1.1: General categories of anti-tumor immunotherapy (clockwise from top). Monoclonal antibodies (mAbs) can be used directly against tumor cells or targeted towards the tumor microenvironment. Direct killing of tumor cells via mAbs is typically through receptor antagonist or agonist activity, but can also target enzymatic activities within the tumor cells. Conjugation of cytotoxic drugs to mAbs is another mechanism of direct tumor killing. These mechanisms can also be applied to the tumor microenvironment. Enhanced immune-mediated killing of tumor cells can also be harnessed through mAbs via enhancement of phagocytosis, antibody-dependent cellular cytotoxicity (ADCC), complement activation, or T cell cross-presentation and activation. **Adoptive transfer of T cells** specific for certain tumor cell antigens can enhance anti-tumor immunity. T cells can be genetically engineered to express T cell receptors that recognize specific tumor cell antigens or tumor-specific autologous T cells can be isolated from the tumor itself with subsequent expansion and reinfusion into the host for the exertion of therapeutic activities. **Therapeutic vaccination** is aimed at redirecting or enhancing immune responses to tumors. Some therapeutic vaccines employ *ex vivo* expansion of autologous antigen-presenting cells (APC) with a common tumor antigen or focus on the modification of tumor cells to express or secrete cytokines that enhance APC activation; in both situations these cells are reinfused into the patient. Other therapeutic vaccines focus on delivery of a specific protein that is overexpressed by the tumor of interest or serves a specific immune function, delivery of an infectious agent to enhance general anti-tumor immunity, or a combination thereof. **Cytokine therapies**, such as IL-2 or the interferons, can be used *in vivo* to enhance immune responses, but can also be employed in the *ex vivo* expansion of immune cells for cell-based therapies. **Modulation of immune signaling** with agents such as BCG and muramyl peptides refers to enhancement of beneficial anti-tumor immune responses or blockade of immunosuppressive signaling. Manipulation of tumor cells to express costimulatory molecules can enhance immune activation, while blockade of inhibitory immune cells (such as T_{regs} or MDSCs) or inhibitory immune receptors (such as CTLA-4) can prevent tumor-based immunosuppression. **Activation of innate immune cells**, such as dendritic cells, can be achieved through *ex vivo* cytokine activation, *in vivo* treatment with toll-like receptor ligands or targeted antigen-loaded antibodies, or even occur naturally secondary to tumor cell death from radiation therapy or chemotherapy**.**

LITERATURE CITED

1. Starkey MP, Scase TJ, Mellersh CS, et al. Dogs really are man's best friend - canine genomics has applications in veterinary and human medicine! Brief Funct Genomic Proteomic 2005;4:112–128.

2. Rowell JL, McCarthy DO, Alvarez CE. Dog models of naturally occurring cancer. Trends Mol Med 2011;17:380–388.

3. Kraus C, Pavard S, Promislow DEL. The size-life span trade-off decomposed: Why large dogs die young. Am Nat 2013;181:492–505.

4. Patterson DF. Companion animal medicine in the age of medical genetics. J Vet Intern Med 2000;14:1–9.

5. Brodey RS, McGrath JT, Reynolds H. A clinical and radiological study of canine bone neoplasms I. J Am Vet Med Assoc 1959;134:53–71.

6. Brodey RS, Sauer RM, Medway W. Canine bone neoplasms. J Am Vet Med Assoc 1963;143:471–495.

7. Brodey RS, Riser WH. Canine osteosarcoma: A clinicopathologic study of 194 cases. Clin Orthop Relat Res 1969;62:54–64.

8. Dorfman SK, Hurvitz AI, Patnaik AK. Primary and secondary bone tumours in the dog. J Small Anim Pract 1977;18:313–326.

9. Ling GV, Morgan JP, Pool RR. Primary bone tumors in the dog: A combined clinical, radiographic, and histologic approach to early diagnosis. J Am Vet Med Assoc 1974;165: 55–67.

10. Withrow SJ, Powers BE, Straw RC, et al. Comparative aspects of osteosarcoma: Dog versus man. Clin Orthop Relat Res 1991;270:159–168.

11. Thompson KG, Pool RR. Tumors of bones. In: Meuten DJ, ed. Tumors in Domestic Animals. Ames, IA: Iowa State Press; 2008:245–318.

12. Ru G, Terracini B, Glickman LT. Host related risk factors for canine osteosarcoma. Vet J 1998;156:31–39.

13. Straw RC, Withrow SJ, Richter SL, et al. Amputation and cisplatin for treatment of canine osteosarcoma. J Vet Intern Med 1991;5:205–210.

14. Heyman SJ, Diefenderfer DL, Goldschmidt MH, et al. Canine axial skeletal osteosarcoma a retrospective study of 116 cases (1986 to 1989). Vet Surg 1992;21:304–310.

15. Brodey RS, Abt DA. Results of surgical treatment in 65 dogs with osteosarcoma. J Am Vet Med Assoc 1976;168:1032–1035.

16. Knecht CD, Priester WA. Musculoskeletal tumors in dogs. J Am Vet Med Assoc 1978;172: 72-74.

17. Misdorp W. Skeletal osteosarcoma. Animal model: Canine osteosarcoma. Am J Path 1980;98:285–288.

18. Misdorp W, Hart A. Some prognostic and epidemiologic factors in canine osteosarcoma. J Natl Cancer Inst 1979;62:537–545.

19. Nielsen SW, Schroder JD, Smith DL. The pathology of osteogenic sarcoma in dogs. J Am Vet Med Assoc 1954;124:28–35.

20. Spodnick GJ, Berg J, Rand WM, et al. Prognosis for dogs with appendicular osteosarcoma treated by amputation alone: 162 cases (1978-1988). J Am Vet Med Assoc 1992;200:995–999.

21. Tjalma RA. Canine bone sarcoma: Estimation of relative risk as a function of body size. J Natl Cancer Inst 1966;36:1137–1150.

22. Wolke RE, Nielsen SW. Site incidence of canine osteosarcoma. J Small Anim Pract 1966;7:489–492.

23. Kansara M, Thomas DM. Molecular pathogenesis of osteosarcoma. DNA Cell Biol 2007;26: 1–18.

24. Janeway KA, Walkley CR. Modeling human osteosarcoma in the mouse: From bedside to bench. Bone 2010;47:859–865.

25. Rainusso N, Wang LL, Yustein JT. The adolescent and young adult with cancer: State of the art - bone tumors. Curr Oncol Rep 2013;15:296–307.

26. Ando K, Heymann MF, Stresing V, et al. Current therapeutic strategies and novel approaches in osteosarcoma. Cancers 2013;5:591–616.

27. Chun R, Kurzman ID, Couto CG, et al. Cisplatin and doxorubicin combination chemotherapy for the treatment of canine osteosarcoma: A pilot study. J Vet Intern Med 2000;14:495–498.

28. Chun R, Garrett LD, Henry C, et al. Toxicity and efficacy of cisplatin and doxorubicin combination chemotherapy for the treatment of canine osteosarcoma. J Am Anim Hosp Assoc 2005;41:382–387.

29. Coley WB. Contribution to the knowledge of sarcoma. Ann Surg 1891;14:199–220.

30. Coley WB. The treatment of malignat tumors by repeated inoculations of erysipelas: With a report of ten original cases. Am J Med Sci 1893;105:487–511.

31. Coley WB. The treatment of inoperable sarcoma by bacterial toxins (the mixed toxins of the Streptococcus erysipelas and the Bacillus prodigiosus). Proc R Soc Med 1910;3:1–48.

32. Cann SAH, van Netten JP, van Netten C. Dr William Coley and tumour regression: A place in history or in the future. Postgrad Med J 2003;79:672–680.

33. Mehl ML, Withrow SJ, Sequin B, et al. Spontaneous regression of osteosarcoma in four dogs. J Am Vet Med Assoc 2001;219:614–617.

34. Thrall DE, Withrow SJ, Powers PE, et al. Radiotherapy prior to cortical allograft limb sparing in dogs with osteosarcoma: A dose response assay. Int J Radiat Oncol Biol Phys 1990;18:1351–1357.

35. Lascelles BDX, Dernell WS, Correa MT, et al. Improved survival associated with postoperative wound infection in dogs treated with limb-salvage surgery for osteosarcoma. Ann Surg Oncol 2005;12:1073–1083.

36. Liptak JM, Dernell WS, Ehrhart N, et al. Cortical allograft and endoprosthesis for limb-sparing surgery in dogs with distal radial osteosarcoma: A prospective clinical comparison of two different limb-sparing techniques. Vet Surg 2006;35:518–533.

37. Culp WTN, Olea-Popelka F, Sefton J, et al. Evaluation of outcome and prognostic factors for dogs living greater than one year after diagnosis of osteosarcoma: 90 cases (1997–2008). J Am Vet Med Assoc 2014;245:1141–1146.

38. Jeys LM, Grimer RJ, Carter SR, et al. Post operative infection and increased survival in osteosarcoma patients: Are they associated? Ann Surg Oncol 2007;14:2887–2895.

39. Lee JA, Kim MS, Kim DH, et al. Postoperative infection and survival in osteosarcoma patients. Ann Surg Oncol 2009;16:147–151.

40. Sottnik JL, U'Ren LW, Thamm DH, et al. Chronic bacterial osteomyelitis suppression of tumor growth requires innate immune responses. Cancer Immunol Immunother 2010;59:367–378.

41. Brodey RS, Fidler IJ, Bech-Nielsen S. Correlation of in vitro immune response with clinical course of malignant neoplasia in dogs. Am J Vet Res 1975;36:75–80.

42. Fidler IJ, Brodey RS, Bech-Nielsen S. In vitro immune stimulation-inhibition to spontaneous canine tumors of various histologic types. J Immunol 1974;112:1051–1060.

43. Bech-Nielsen S, Reif JS, Brodey RS. Pre-and postoperative studies of in vitro cell-mediated reactivity in canine osteosarcoma. Am J Vet Res 1978;39:87–93.

44. Bech-Nielsen S, Brodey RS, Fidler IJ, et al. The effect of BCG on in vitro immune reactivity and clinical course in dogs treated surgically for osteosarcoma. Eur J Cancer 1977;13:33–41.

45. Segal-Eiras A, Robins RA, Hannant D, et al. Circulating immune complexes in dogs with osteosarcoma. Br J Cancer 1982;46:444–447.

46. Haines DM, Bruland OS. Immunohistochemical detection of osteosarcoma-associated antigen in canine osteosarcoma. Anticancer Res 1989;9:903–908.

47. Haines DM, Bruland OS, Matte G, et al. Immunoscintigraphic detection of primary and metastatic spontaneous canine osteosarcoma with F(ab')2 fragments of osteosarcoma-associated monoclonal antibody TP-1. Anticancer Res 1992;12:2151–2158.

48. Page RL, Garg PK, Garg S, et al. PET imaging of osteosarcoma in dogs using a fluorine-18-labeled monoclonal antibody Fab fragment. J Nucl Med 1994;35:1506–1513.

49. Biller BJ, Elmslie RE, Burnett RC, et al. Use of FoxP3 expression to identify regulatory T cells in healthy dogs and dogs with cancer. Vet Immunol Immunopathol 2007;116:69–78.

50. O'Neill K, Guth A, Biller B, et al. Changes in regulatory T cells in dogs with cancer and associations with tumor type. J Vet Intern Med 2009;23:875–881.

51. Rissetto KC, Rindt H, Selting KA, et al. Cloning and expression of canine CD25 for validation of an anti-human CD25 antibody to compare T regulatory lymphocytes in healthy dogs and dogs with osteosarcoma. Vet Immunol Immunopathol 2010;135:137–145.

52. Biller BJ, Guth A, Burton JH, et al. Decreased ratio of CD8+ T cells to regulatory T cells associated with decreased survival in dogs with osteosarcoma. J Vet Intern Med 2010;24:1118–1123.

53. Sherger M, Kisseberth W, London C, et al. Identification of myeloid derived suppressor cells in the peripheral blood of tumor bearing dogs. BMC Vet Res 2012;8:209–220.

54. Goulart MR, Pluhar GE, Ohlfest JR. Identification of myeloid derived suppressor cells in dogs with naturally occurring cancer. PLoS One 2012;7:e33274.

55. Sottnik JL, Rao S, Lafferty MH, et al. Association of blood monocyte and lymphocyte count and disease-free interval in dogs with osteosarcoma. J Vet Intern Med 2010;24:1439–1444.

56. Selmic LE, Lafferty MH, Kamstock DA, et al. Outcome and prognostic factors for osteosarcoma of the maxilla, mandible, or calvarium in dogs: 183 cases (1986-2012). J Am Vet Med Assoc 2014;245:930–938.

57. Wasserman J, Diese L, VanGundy Z, et al. Suppression of canine myeloid cells by soluble factors from cultured canine tumor cells. Vet Immunol Immunopathol 2010;145:420–430.

58. Luca S, Mihaescu T. History of BCG vaccine. Maedica 2013;8:53–58.

59. Gandhi NM, Morales A, Lamm DL. Bacillus Calmette-Guerin immunotherapy for genitourinary cancer. BJU Int 2013;112:288–297.

60. Kawai K, Miyazaki J, Joraku A, et al. Bacillus Calmette-Guerin (BCG) immunotherapy for bladder cancer: Current understanding and perspectives on engineered BCG vaccine. Cancer Sci 2013;104:22–27.

61. Owen LN, Bostock DE. Effects of intravenous BCG in normal dogs and in dogs with spontaneous osteosarcoma. Eur J Cancer 1974;10:775–780.

62. Owen LN, Bostock DE, Lavelle RB. Studies on chemotherapy and immunotherapy in canine lymphosarcoma and osteosarcoma. Bibl Haematol 1975;43:522–523.

63. Betton GR, Gorman NT, Owen LN. Cell mediated cytotoxicity in dogs following systemic or local BCG treatment alone or in combination with allogeneic tumour cell lines. Eur J Cancer 1979;15:745–754.

64. Betton GR, Gorman NT. Cell-mediated responses in dogs with spontaneous neoplasms. I. Detection of cell-mediated cytotoxicity by the chromium-51 release assay. J Natl Cancer Inst 1978;61:1085–1093.

65. Gorman NT. Alveolar macrophage cytotoxicity in dogs following intravenous BCG. Eur J Cancer 1979;15:1051–1059.

66. Ugel S, Peranzoni E, Desantis G, et al. Immune tolerance to tumor antigens occurs in a specialized environment of the spleen. Cell Rep 2012;2:628–639.

67. Meyer JA, Dueland RT, MacEwen EG, et al. Canine osteogenic sarcoma treated by amputation and MER: An adverse effect of splenectomy on survival. Cancer 1982;49:1613–1616.

68. Schroit AJ, Fidler IJ. Effects of liposome structure and lipid composition on the activation of the tumoricidal properties of macrophages by liposomes containing muramyl dipeptide. Cancer Res 1982;42:161–167.

69. Kleinerman ES, Jia SF, Griffin J, et al. Phase II study of liposomal muramyl tripeptide in osteosarcoma: The cytokine cascade and monocyte activation following administration. J Clin Oncol 1992;10:1310–1316.

70. Kleinerman ES, Murray JL, Snyder JS, et al. Activation of tumoricidal properties in monocytes from cancer patients following intravenous administration of liposomes containing muramyl tripeptide phosphatidylethanolamine. Cancer Res 1989;49:4665–4670.

71. Asano T, McWatters A, An T, et al. Liposomal muramyl tripeptide up-regulates interleukin-1 alpha, interleukin-1 beta, tumor necrosis factor-alpha, interleukin-6 and interleukin-8 gene expression in human monocytes. J Pharmacol Exp Ther 1994;268:1032–1039.

72. Kleinerman ES, Gano JB, Johnston DA, et al. Efficacy of liposomal muramyl tripeptide (CGP 19835A) in the treatment of relapsed osteosarcoma. Am J Clin Oncol 1995;18:93–99.

73. Meyers PA, Schwartz CL, Krailo M, et al. Osteosarcoma: A randomized, prospective trial of the addition of ifosfamide and/or muramyl tripeptide to cisplatin, doxorubicin, and high-dose methotrexate. J Clin Oncol 2005;23:2004–2011.

74. Smith BW, Kurzman ID, Schultz KT, et al. Muramyl peptides augment the in vitro and in vivo cytostatic activity of canine plastic-adherent mononuclear cells against canine osteosarcoma cells. Cancer Biother Radiopharm 1993;8:137–144.

75. Kurzman ID, Shi F, MacEwen EG. In vitro and in vivo canine mononuclear cell production of tumor necrosis factor induced by muramyl peptides and lipopolysaccharide. Vet Immunol Immunopathol 1993;38:45–56.

76. Shi F, MacEwen EG, Kurzman ID. In vitro and in vivo effect of doxorubicin combined with liposome-encapsulated muramyl tripeptide on canine monocyte activation. Cancer Res 1993;53:3986–3991.

77. Kurzman ID, Shi F, Vail DM, MacEwen EG. In vitro and in vivo enhancement of canine pulmonary alveolar macrophage cytotoxic activity against canine osteosarcoma cells. Cancer Biother Radiopharm 1999;14:121–128.

78. MacEwen EG, Kurzman ID, Rosenthal RC, et al. Therapy for osteosarcoma in dogs with intravenous injection of liposomeencapsulated muramyl tripeptide. J Natl Cancer Inst 1989;81:935–938.

79. Kurzman ID, MacEwen EG, Rosenthal RC, et al. Adjuvant therapy for osteosarcoma in dogs: Results of randomized clinical trials using combined liposome-encapsulated muramyl tripeptide and cisplatin. Clin Cancer Res 1995;1:1595–1601.

80. Mitchell DH, Withrow SJ, Johnston MR, et al. Cytotoxicity against autologous, allogeneic, and xenogeneic tumor targets by human recombinant interleukin-2-activated lymphocytes from healthy dogs and dogs with lung tumors. Am J Vet Res 1991;52:1132–1136.

81. Ohnishi H, Okuno K, Yasutomi M. Successful in vivo generation of canine lymphokine-activated killer cells by continuous recombinant interleukin-2 infusion through the splenic artery. Cancer Biother Radiopharm 1993;8:213–222.

82. Khanna C, Hasz DE, Klausner JS, et al. Aerosol delivery of interleukin 2 liposomes is nontoxic and biologically effective: Canine studies. Clin Cancer Res 1996;2:721–734.

83. Khanna C, Anderson PM, Hasz DE, et al. Interleukin-2 liposome inhalation therapy is safe and effective for dogs with spontaneous pulmonary metastases. Cancer 1997;79:1409–1421.

84. Dow S, Elmslie R, Kurzman I, et al. Phase I study of liposome-DNA complexes encoding the interleukin-2 gene in dogs with osteosarcoma lung metastases. Hum Gene Ther 2005;16:937-946.

85. Visonneau S, Cesano A, Jeglum KA, et al. Adjuvant treatment of canine osteosarcoma with the human cytotoxic T-cell line TALL-104. Clin Cancer Res 1999;5:1868–1875.

86. Mata M, Vera JF, Gerken C, et al. Toward immunotherapy with redirected T cells in a large animal model: Ex vivo activation, expansion, and genetic modification of canine T cells. J Immunother 2014;37:407–415.

87. Koshkina NV, Khanna C, Mendoza A, et al. Fas-negative osteosarcoma tumor cells are selected during metastasis to the lungs: The role of the Fas pathway in the metastatic process of osteosarcoma. Mol Cancer Res 2007;5:991–999.

88. Gordon N, Koshkina NV, Jia SF, et al. Corruption of the Fas pathway delays the pulmonary clearance of murine osteosarcoma cells, enhances their metastatic potential, and reduces the effect of aerosol gemcitabine. Clin Cancer Res 2007;13:4503–4510.

89. Modiano JF, Bellgrau D, Cutter GR, et al. Inflammation, apoptosis, and necrosis induced by neoadjuvant Fas ligand gene therapy improves survival of dogs with spontaneous bone cancer. Mol Ther 2012;20:2234–2243.

90. Rodriguez CO Jr, Crabbs TA, Wilson DW, et al. Aerosol gemcitabine: Preclinical safety and in vivo antitumor activity in osteosarcoma-bearing dogs. J Aerosol Med Pulm Drug Deliv 2010;23:197–206.

91. Thamm DH, Kurzman ID, King I, et al. Systemic administration of an attenuated, tumor-targeting Salmonella typhimurium to dogs with spontaneous neoplasia: Phase I evaluation. Clin Cancer Res 2005;11:4827–4834.

92. Gentschev I, Patil SS, Adelfinger M, et al. Characterization and evaluation of a new oncolytic vaccinia virus strain LIVP6.1.1 for canine cancer therapy. Bioengineered 2013;4:84–89.

93. Finocchiaro LME, Villaverde MS, Gil-Cardeza ML, et al. Cytokine-enhanced vaccine and interferon-beta plus suicide gene as combined therapy for spontaneous canine sarcomas. Res Vet Sci 2001;91:230–234.

94. Peruzzi D, Mesiti G, Ciliberto G, et al. Telomerase and HER-2/neu as targets of genetic cancer vaccines in dogs. Vaccine 2010;28:1201–1208.

CHAPTER II

CHARACTERIZING THE EXPRESSION AND FUNCTIONALITY OF TOLL-LIKE RECEPTORS IN IMMUNE AND OSTEOSARCOMA CELL LINES

Abstract

Toll-like receptors (TLRs) are considered the critical bridge between the innate and adaptive immune system. Investigations surrounding TLRs and their respective agonists in regard to cancer-based immunotherapy is not new. Indeed, TLRs have shown to function in both pro- and anti-tumorigenic capacities depending on the specific tumor histology investigated, showing the dichotomous nature of how these receptors influence cancer. However, little research into TLRs for the treatment of either human or canine OS has been published, yet TLRs and their respective agonists constitute an attractive area of immunotherapy from a translational perspective, given their ability to be used across-species without substantial modification, which is often not the case with passive immunotherapies such as mAbs. Therefore, we chose to employ a top-down, systematic approach to identify TLR agonist(s) that would maximize anti-tumor immunity while minimizing OS pro-inflammatory tumorigenesis, with the ultimate goal of identifying a TLR agonist with the potential to be employed within pet dogs with spontaneous OS.

This chapter elaborates on the preliminary studies that identified the TLR agonists Pam3CSK4 (TLR1/2), Poly(I:C) (TLR3), and CpG ODN 2395 (TLR9) as potential immunotherapeutics to employ within **Chapter III**, which utilizes a clinically-relevant murine model of metastatic OS. We show that TLRs exhibit a dichotomous nature in regard to mRNA expression, that cellular viability is also variably influenced by different TLR agonists, and even classical inflammatory and pro-tumorigenic cytokine secretion differs depending on the agonist

investigated. To round-out these studies, we integrated the evaluation of both murine and canine immune and OS cells to develop a better understanding of how different TLR agonists would function in a multi-species capacity. Furthermore, our investigations suggested that out of our 3 candidate TLR agonists, the TLR9 agonist CpG ODN 2395 may be least pro-tumorigenic in an OS setting while still promoting a meaningful anti-tumor immune response.

Introduction

TLRs are a class of type I transmembrane proteins that function as pattern recognition receptors (PRRs), which recognize conserved pathogen-associated molecular patterns (PAMPs) from various bacteria, fungi, protozoa, parasites, and viruses.[1,2] TLRs are classically recognized to be expressed by macrophages and dendritic cells, key players in the innate immune system, but TLRs can also be expressed by surface epithelium and mesenchymal tissue, along with B and T cells of the adaptive immune system.[1,3-5] Numerous cancer histologies have also been shown to express TLRs, whether at normal tissue levels or in an aberrant nature.[6-8]

The subcellular location of TLRs varies, with TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 localized to the cytoplasmic membrane while TLR3, TLR7, TLR8, TLR9, TLR11, TLR12, and TLR13 are found within the endosome. To date, the human-expressed TLRs include TLR1-10, while murine cells express TLR1-9 and TLR11-13.[1,9] Various TLRs have also been shown to be expressed by canine cells.[10-15]

Subcellular localization is dictated by the specific class of pathogen recognized by each individual TLR. For example, cytoplasmic membrane TLRs most often recognize bacterial-associated PAMPS, while endosomal TLRs predominate with RNA (viral) or DNA (viral/bacterial) recognition; however, most TLRs are not restricted to recognizing only one class

of pathogen. In addition to differing in their cognate ligands, signaling pathways for TLRs exhibit divergence, utilizing various TIR domain-containing adaptors such as MyD88, TRAM, TRIF, or TIRAP. These various adaptors ultimately result in the activation of the MAPK pathway, along with additional downstream activation of specific transcription factors including NFκB or interferon regulatory factors (IRFs). Resultantly, TLR ligation and activation results in pro-inflammatory cytokine and/or type I IFN secretion. This innate immune response further shapes the adaptive immune system, resulting in system-wide pathogen defense.[1,9,16,17]

From an anti-tumor immunity standpoint, TLRs and their agonists are ideal candidates for the study and application of novel immunotherapeutics, given their conserved nature,[18] ability to activate innate cell killing,[19] and fine-tuning of the adaptive immune response,[16] all of which are desirable traits in an immunotherapeutic. However, TLRs have been shown to promote tumorigenesis through inflammation, yielding a so-called "double-edged sword" to this immunotherapeutic strategy, whereby pro-inflammatory tumorigenesis may override anti-tumor immunity.[20] As the current evaluation of TLRs for the treatment of OS in both dogs and people is very limited, this "double-edge" theory led us to initially determine the expression and functionality amongst multiple TLRs and their respective agonists comparatively between innate immune and OS cells, in an effort to shift the balance toward anti-tumor immunity and away from pro-inflammatory tumorigenesis. We hypothesized that there would be divergence between expression and functionality between immune and OS cells, allowing for the selection of 2-3 TLR agonists that could preferentially shift the balance away from tumorigenesis and towards anti-tumor immunity.

Our findings support our hypothesis, showing that TLRs do exhibit some level of variability in expression between immune and OS cells, while agonism with various ligands can

either enhance or decrease cellular viability. Based on this expression and functionality data, we choose to further investigate Pam3CSK4 (TLR1/2), Poly(I:C) (TLR3), and CpG ODN 2395 (TLR9) based upon their promising differentials within these assays. These 3 agonists were then subjected to measures of pro-inflammatory and pro-tumorigenic cytokine secretion, along with investigation of pro-tumorigenic protein upregulation. Here we show all three agonists upregulated various pro-inflammatory cytokines that bolster anti-tumor immunity, but exhibited more discriminatory divergence in the production of certain pro-tumorigenic cytokines, such as IL-6, IL-8, and VEGF. While none of these agonists appeared to strictly shift the balance solely towards tumorigenesis, CpG ODN 2395 (TLR9) was identified as having the best potential for initiating pro-inflammatory anti-tumor immunity with concurrent minimal upregulation of a pro-tumorigenic environment in the context of OS.

Materials and Methods

Cell Lines

Murine immune cell lines D2SC/1, J774, and RAW and clonally-related[21] osteosarcoma cell lines K7M2 and K12 were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S) at 37ºC and 5% CO_2 in a humidified incubator and passaged as needed. Canine immune cell lines DH82, Nike, and 030-D and osteosarcoma cell lines Abrams, HMPOS, and K003 were cultured using the same method.

Toll-Like Receptors Agonists

TLR agonists for TLRs 2, 3, 4, 7, and 9 were purchased from InvivoGen (San Diego, CA) and reconstituted in endotoxin-free water. A complete list of the TLR agonists investigated is provided in **Table 2.1**.

MTS Assays for Initial TLR Agonist Screening

Immune and OS cells were plated at 5-10,000 cells/well and allowed to adhere overnight. Media was then replaced with new media containing various TLR agonists (**Table 2.1**) at 3-4 different concentrations per agonist. Concentrations investigated for each ligand were based upon TLR activation ranges provided by InvivoGen. After 20 hours of treatment, media supernatants were removed, and cells were analyzed via colorimetric MTS measurement with the CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Madison, WI) to quantify cellular viability in response to TLR agonist treatment.

Enzyme-Linked Immunosorbent Assays

Commercially available enzyme-linked immunosorbent assays (ELISAs) were purchased for cytokine assessment. Murine-specific IL-6, IL-12 p70, TNFα, and VEGF were measured with Quantikine ELISA (R&D Systems, Minneapolis, MN). Murine-specific IFNα was measured with VeriKine ELISA (PBL Assay Science, Piscataway, NJ). Murine-specific IL-8 was measured with Mouse IL-8 ELISA (MyBioSource, San Diego, CA). Canine-specific IL-6, IL-8, and TNFα were measured with Quantikine ELISA and IL-12/IL-23 p40 was measured with DuoSet ELISA (R&D Systems). Canine-specific IFNα was measured with Canine IFNα ELISA (MyBioSource).

Murine and canine cell lines were plated in quadruplicate at a density of 10,000 cells/well in DMEM supplemented with 10% FBS and 1% P/S and allowed to adhere overnight. The next day, the media was replaced with new media containing either Pam3CSK4, Poly(I:C), or CpG ODN 2395 at varying concentrations (0.3, 1, or 10 μg/mL for Pam3CSK4; 10, 30, or 100 μg/mL for Poly(I:C); 1, 25, or 50 μg/mL for CpG ODN 2395). After a 20-hour incubation, the plates were centrifuged for 5 minutes to settle any floating cells, then media supernatants were collected and frozen back at -80ºC until ELISA analysis was performed. After media removal, adherent cells were analyzed via MTS assay (Promega) for normalization of ELISA results.

Real-Time PCR (qPCR)

Total RNA was extracted from cells harvested from confluent petri dishes using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions for animal cells. Total RNA extraction was followed with DNase treatment using either an RNase-free DNase Set (Qiagen) or the TURBO DNA-*free* Kit (Invitrogen, Carlsbad, CA). DNase-treated total RNA samples were purified via ethanol precipitation. RNA was then quantified and assessed for purity with the NanoDrop 1000 or the NanoDrop One (Thermo Scientific, Waltham, MA). RNA integrity was measured using the Agilent 2100 Bioanalyzer (Santa Clara, CA). All RNA samples chosen for cDNA synthesis had an A260/280 ratio >1.8, A260/230 ratio <2.4, and RNA integrity number (RIN) >8.

Following quantification and RIN analysis, 2 μg of RNA was reverse-transcribed to cDNA with the SuperScript or SuperScript III First Strand Synthesis System (Invitrogen) for murine and canine samples, respectively, using random hexamers. No reverse transcriptase (-RT) controls were also prepared for each sample. 25 ng of the resultant cDNA was used in a 25 μL-PCR

reaction composed of 12.5 µL TaqMan Universal Master Mix II with UNG (Applied Biosystems, Foster City, CA), 6.25 µL water, and 1.25 µL TaqMan Gene Expression Assay (Applied Biosystems). The TaqMan Gene Expression Assays consist of pre-mixed primers and the TaqMan MGB probe at a 20x concentration. Murine and canine genes of interest (GOI) and corresponding assay numbers are listed in **Tables 2.2** and **2.3**, respectively.

For each GOI, three biological replicates from each cell line were run in triplicate on an Applied Biosystems 7500 Real-Time PCR System. No template controls were also utilized during each run to assess for genomic DNA contamination within the PCR mixture. Negative RT controls were run in triplicate for each sample at least once with a primer/probe set that could detect genomic DNA, to ensure the efficacy of DNase treatment. For canine TLR3, the amplification efficiency of the custom primer/probe set (AJWR2PO) was determined to be 96%, calculated from the slope of a standard curve generated from a 2-fold dilution series. The run method for all reactions included 2 minutes at 50°C for UNG incubation, 10 minutes at 95°C for polymerase activation, followed by 40 cycles of denaturing at 95°C for 15 seconds and annealing/extending at 60°C for 1 minute. Normfinder, a free Excel add-in, was used to determine the most stable reference gene for each species.[22] ΔC_T was calculated for each biological replicate by the equation $\Delta C_T = C_T$ target - C_T reference. Based off Normfinder stability values, GAPDH was used for the reference gene in both species.

Western Blot Analysis

K7M2, K12, and Abrams cell lines were grown to 80% confluence in petri dishes and treated with either Pam3CSK4, Poly(I:C), or CpG ODN 2395 for 20 hours, at the same concentrations utilized for ELISA cytokine measurement. After 20 hours, cell pellets were

collected and protein was extracted using M-PER Mammalian Protein Extraction Reagent (Thermo Scientific). Protein levels were determined with the Pierce BCA Protein Assay Kit (Thermo Scientific). Based off protein measurements, 50 μg protein from each cell line and treatment concentration were resolved using 4-20% SDS-PAGE (Mini-PROTEAN TGX Pre-cast Gels, Bio-Rad, Hercules, CA) and transferred onto nitrocellulose or PVDF membranes. Membranes were blocked with 5% non-fat dry milk powder in tris-buffered saline/Tween 20 (TBS-T) for 1 hour at room temperature, then incubated with primary antibody for 16 hours at 4°C. Primary antibodies for Bcl-2 (BD610538) were utilized at a dilution of 1:500 for murine cells and 1:1000 for canine cells. XIAP (cs2042) and pAkt (cs9271) were utilized at a dilution of 1:500 and 1:1000, respectively, for both species.

Primary antibody incubation was followed by three washes in TBS-T, then HRP-conjugated secondary antibody was added for 1 hr at room temperature at 1:1000 in 5% milk + TBS-T. Three additional washes in TBS-T were performed prior to imaging using an enhanced chemiluminescent (ECL) substrate (SuperSignal West Femto Maximum Sensitivity Substrate, Thermo Scientific) and the ChemiDoc XRS+ System (Bio-Rad). Band volume analysis was performed using Image Lab software (Bio-Rad). Bcl-2 and XIAP bands were normalized to β-actin (ab6276, 1:5000 dilution). pAkt bands were normalized to Akt (cs9272; 1:1000 dilution). Protein expression for each treatment concentration was determined and expressed as a percent qualitative change (±50%) relative to the untreated control.

Statistical Analysis

Statistical analysis was performed with GraphPad Prism version 7.0a for Mac OS X (GraphPad Software, La Jolla, CA). Significance was set at $p < 0.05$. qPCR, MTS, and ELISA

data are expressed as the mean ± standard error of the mean (SEM). Normality was assessed with the Shapiro-Wilk test. One-way ANOVA with Tukey's was used for parametric qPCR data and the Kruskal-Wallis test with Dunn's was used for non-parametric qPCR data. MTS assay data was assessed with 2-way ANOVA without matching and Dunnett's. Parametric ELISA data was analyzed with one-way ANOVA with Dunnett's and non-parametric data with the Kruskal-Wallis test with Dunn's. For all data, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

Results

TLRs Exhibit Differential Expression in Immune and OS Cells

While TLR expression has been extensively evaluated in numerous cancer histologies, minimal investigation of TLR expression in osteosarcoma has been published to date.[23-27] Therefore, we sought to determine if differential expression between immortalized immune and OS cells were distinguishable by qPCR, as dichotomous expression between the immune system and OS may help guide a rational therapeutic choice of TLR agonist. To this extent, we performed baseline TLR mRNA expression levels using Taqman qPCR on immune and OS cells of murine and canine origin (**Figures 2.1 and 2.2**). Murine immune and OS cells exhibited no difference in ΔC_T for TLR2, TLR4, and TLR7 (**Figure 2.1a, d, e**). However, when analyzed as a group, murine OS cells (K7M2 and K12) had lower expression of TLR2 (characterized by increased ΔC_T) when compared to immune cells (**Figure 2.1b**). TLR3 expression was not different between most murine immune and OS cell comparisons, except for RAW and K7M2, where RAW (average ΔC_T =13) had decreased expression of TLR3 comparatively to K7M2 (average ΔC_T =7) (**Figure 2.1c**). Both K7M2 and K12 also failed to have measurable C_T values for TLR9, while the immune cells had abundant expression of TLR9 with low ΔC_T (**Figure 2.1f**).

For canine immune cells, the Nike cell line did have not measurable C_T values for any GOI except RPS18, a potential reference gene, and a single Nike biological replicate had a high C_T of 37 for TLR9 (data not shown). PCR results from 030-D are also not shown, as although GAPDH was the most stable reference gene overall, 030-D had on average a 23-fold increase in expression of GAPDH relative to the other cell lines, which skewed group-wise comparisons. TLR C_T values for 030-D, however, were often within 1-2 cycles of DH82 values. Decreased expression of TLR2 and TLR4 was observed for canine OS cells in comparison to the immune cell line DH82 for 1-2 of the three canine OS cell lines screened (Abrams and/or K003) (**Figure 2.2a, c**). Trends towards decreased expression of TLR3 and TLR7 were also noted for the OS cell lines, but were not statistically significant (**Figure 2.2b, d**). TLR9 expression did not differ between canine immune and OS cells (**Figure 2.2e**). From these results, we concluded that neither murine nor canine OS cells consistently express aberrantly high levels of TLRs compared to immune cells of the same species, although differential expression was occasionally observed between immune and OS cells. This differential expression was most apparent with TLR2 and TLR9, which were flagged as potential targets. This was based off the consistent increase in TLR2 ΔC_T for OS cells across both species (equating to less expression of TLR2 comparatively to the immune cells), and the lack of measurable TLR9 expression in murine OS in combination with stable TLR9 expression for canine OS.

TLR Agonists Variably Influence Cellular Proliferation

qPCR results identified TLR2 and TLR9 as promising targets for potential immunotherapy in OS; however, mRNA expression does not always correlate to protein expression and also does not indicate the level of receptor functionality. Therefore, we sought to assess the influence of

TLR agonists on OS cellular proliferation and metabolism, in order to identify potential agonists that may not be directly stimulatory to OS, but could still have the potential to exert positive immunobiological effects.

To assess this, we screened multiple TLR agonists *in vitro* (**Table 2.1**) in both murine and canine immune and OS cells utilizing MTS optical density (OD) as indicative of changes in cellular viability after 20 hours of TLR agonist incubation (**Figures 2.3 and 2.4**). Results were highly variable between immune and OS cells of both species. Murine immune cells D2SC/1 and RAW responded most robustly to CpG ODN 2395 (TLR9) incubation, with significantly increased OD levels at all 3 concentrations (1, 25, and 50 μg/mL) investigated compared to untreated controls (**Figure 2.3h**). RAW immune cells also expressed higher OD with LTA (TLR2) and Imiquimod (TLR7) treatment (**Figure 2.3b, f**). All other TLR agonists exhibited either stable or decreased OD for murine immune cells (**Figure 2.3a, c-e, g**). Comparatively, CpG ODN 2395 at 50 μg/mL was the only TLR agonist to elevate OD in the canine immune cell line DH82 (**Figure 2.4h**). These findings suggest that the TLR9 agonist CpG ODN 2395 has the most widespread ability to influence cellular proliferation in immune cells of multiple origins.

OS cells also exhibited variable responses secondary to TLR agonism. For CpG ODN 2395, no change in OD was observed for either murine or canine OS (**Figures 2.3h and 2.4h**). Pam3CSK4 (TLR1/2) caused significant decreases in OD for both murine and OS cell lines at the highest concentration of 10 μg/mL (**Figure 2.3a and 2.4a**). Poly(A:U) and Poly(I:C), both TLR3 ligands, caused variable, often dose-dependent decreases for murine OS (**Figure 2.3c, d)**. This effect was also sustained for Poly(A:U) in canine OS (**Figure 2.4c**), but was not observed in canine OS with Poly(I:C) (**Figure 2.4d**). Imiquimod decreased OD in both murine OS lines (**Figure 2.3f**), but remained stable overall amongst canine OS (**Figure 2.4f**). LTA, LPS (TLR4), and *E.*

coli ssDNA (TLR9) agonism resulted in either elevated or stable OD amongst the majority of murine and canine OS cell lines (**Figures 2.3b, e, g, and 2.4b, e, g**). When evaluating which agonists had a positive viability or proliferative effect on immune cells while concurrently exhibiting a predominantly stable or inhibitory effect on OS cells across both species, the findings identified Pam3CSK4 (TLR1/2), Poly(A:U)/Poly(I:C) (TLR3), and CpG ODN 2395 (TLR9) as possible candidates for further evaluation.

Based upon collective MTS assay and qPCR results, we determined that Pam3CSK4 (TLR1/2), Poly(I:C) (TLR3), and CpG ODN (TLR9) were all reasonable candidates for further evaluation as potential therapeutics for metastatic OS. While Poly(I:C) did not exhibit as profound inhibitory effects as Poly(A:U) within the MTS assay, it was chosen over Poly(A:U) due to its stronger immunogenicity.

TLR1/2, 3, and 9 Agonists Induce Robust TNFα Secretion in Immune Cells

To examine the potential immunobiologic activity of our 3 selected TLR agonists, we quantified secretion of several important immunological cytokines by our murine (D2SC/1, J774, RAW) and canine (DH82, Nike, 030-D) immune cell lines. IL-12, IFNα, and TNFα were measured via species-specific ELISA in response to a 20 hour incubation with the TLR agonists Pam3CSK4 (TLR1/2), Poly(I:C) (TLR3), and CpG ODN 2395 (TLR9). Murine immune cells exhibited robust secretion of TNFα in response to all 3 agonists (**Figure 2.5**). This secretion was consistently dose-dependent for all lines, except RAW, which in response to Pam3CSK4 and CpG ODN 2395 exhibited maximal secretion levels at the lowest concentration levels evaluated (0.3 μg/mL and 1 μg/mL, respectively). DH82 secreted elevated levels of TNFα in response to Pam3CSK4 and Poly(I:C) at all three concentrations tested, these levels were dose-dependent for

Poly(I:C) but not for Pam3SK4. Measurable TNFα levels, however, were only detected at the highest concentration of CpG ODN 2395 (50 μg/mL) for DH82. No statistical change in measurable TNFα was observed for 030-D with either Pam3CSK4 or CpG ODN 2395, but significant increases were noted with Poly(I:C) treatment (**Figure 2.6**). Nike failed to secrete TNFα under normal conditions or in the presence of any agonist (data not shown).

IL-12 secretion was not detectable in any of the canine immune cells, regardless of treatment, and was not detectable in response to Pam3CSK4 within the murine immune cells (data not shown). IL-12 was only elevated in response to Poly(I:C) treatment at high doses within the RAW murine cell line, whereas CpG ODN 2395 decreased secretion. For D2SC/1 and J774, IL-12 secretion was either stable or decreased with Poly(I:C) and CpG ODN 2395 (**Figure 2.7**). IFNα was also not detected in response to Pam3CSK4 and CpG ODN 2395 in the murine immune cells (data not shown), but was increased in J774 and RAW cells with Poly(I:C). D2SC/1 did not secrete IFNα (**Figure 2.8**). Elevations in IFNα in the canine cell lines was minimal in response to all three TLR agonists (**Figure 2.9**).

CpG ODN 2395 Exerts Minimal Pro-Tumorigenic Activity In Vitro

In addition to evaluating potential immunobiologic activity of our 3 selected TLR agonists, it was equally important to evaluate the pro-tumorigenic activity of these agonists within OS cells, the intended therapeutic target. To this extent, we chose to evaluate the secretion of several cytokines (IL-6, IL-8, and VEGF) and the expression levels of proteins (Bcl-2, XIAP, pAkt) central to OS tumorigenesis,[28] in response to a 20 hour incubation with the selected agonists.

All 5 OS cell lines previously evaluated (murine OS cell lines K7M2 and K12; canine OS cell lines Abrams, HMPOS, and K003) were included in the evaluation of pro-tumorigenic

cytokine secretion in response to TLR agonism. The murine OS cells, K7M2 (**Figure 2.10a**) and K12 (**Figure 2.10b**), responded similarly to Pam3CSK4 treatment with stable production of IL-8 and increased production of VEGF, but had variable production of IL-6 (increased for K7M2 and decreased to steady for K12). Poly(I:C) treatment also stimulated production of IL-6 and IL-8 in both murine OS lines, but was inhibitory for K7M2 VEGF secretion at elevated concentrations and stimulatory for K12. CpG ODN 2395 therapy resulted in either stable (IL-6, IL-8) or reduced (VEGF) production in K7M2. K12 cells responded with initial peaks in secretion for IL-6 and VEGF, followed by a return to basal secretion levels at doses of 25 and 50 μg/mL. For IL-8, however, K12 cells exhibited increased secretion amongst all treatment levels but the absolute increase in IL-8 concentrations remained minimal (less than 30 pg/mL).

Pam3CSK4 also consistently induced elevated production of all 3 cytokines amongst the Abrams (**Figure 2.11a**), HMPOS (**Figure 2.11b**), and K003 (**Figure 2.11c**) canine OS cell lines. Similarly, Poly(I:C) treatment induced secretion of IL-6 and IL-8 amongst the canine OS cells except for K003, which had stable secretion of IL-6 regardless of Poly(I:C) concentration. VEGF secretion elevated in response to low-levels of Poly(I:C) in Abrams, but remained stable to decreased in HMPOS and K003. CpG ODN 2395 did not increase IL-6, IL-8, or VEGF levels, with the exception of IL-6 in HMPOS, which went from undetectable to detectable with CpG ODN 2395 treatment. This conversion to detectable secretion was not statistically significant.

For pro-tumorigenic protein expression (**Figure 2.12**), data was compared qualitatively to untreated controls for K7M2, K12, and Abrams. The K12 murine OS cell line was the only cell line to have an elevation >50% of baseline expression levels (74% increase in Bcl-2 in response to 10 μg/mL of Pam3CSK4). The majority of changes, however, were within ±50% of untreated baseline expression levels and were not considered significant. Few scenarios resulted in a

decrease >50% of baseline. This included a 59% reduction in pAkt for Abrams treated with Pam3CSK4 (1 and 10 μg/mL), a 60% (100 μg/mL) and 62% (30 μg/mL) reduction in XIAP for K7M2 and K12, respectively, with Poly(I:C) treatment, and a 75% reduction in pAkt for K7M2 with CpG ODN 2395. The K12 cell line also demonstrated a 74% and 69% reduction in pAkt following high-doses of CpG ODN 2395 (25 and 50 μg/mL, respectively).

Discussion

While the findings from this chapter were informative and support our hypothesis of divergence between expression and functionality amongst various TLRs and their respected agonists, overall directionality of the data (both between and within species) was not uniform. This lack of uniformity was not unexpected, and when we concentrated on the most salient features of each methodology employed, important trends do appear.

In the murine cell lines evaluated, we were unable to produce TLR9 amplicons with qPCR for both the K7M2 (metastatic) and K12 (non-metastatic) cell lines, while the immune cell lines had high levels of mRNA expression. This could be due to our Taqman assay not having adequate TLR9 transcript coverage, but unfortunately, no alternative Taqman assays for murine TLR9 are currently available. However, this finding does raise the possibility that TLR9 protein expression in the murine OS cell lines is minimal, which might negate pro-tumorigenic activities of TLR9 agonism *in vivo*. This, while only theoretical, would be highly beneficial from a therapeutic standpoint. Although the canine OS cell lines did have measurable TLR9 mRNA levels, their expression levels were not different from the immune cells, suggesting TLR9 levels in canine OS are not aberrantly upregulated.

Cytokine secretion by the murine immune cell lines was not discriminatory between TLR2, TLR3, and TLR9, as all three agonists amplified TNFα, and exhibited only random and marginal effects on other important immune cytokines (IL-12 and IFNα). When also evaluating pro-tumorigenic cytokine secretion by K7M2, CpG ODN 2395 appeared most favorable from a strict tumorigenesis angle, but Poly(I:C) also had minimal pro-tumorigenic cytokine effects and was more pan-immunostimulatory than CpG ODN 2395. Interestingly, when evaluating pro-tumorigenic protein expression, Poly(I:C) elevated pAkt in K7M2, whereas CpG ODN 2395 decreased pAkt. When including murine mRNA expression levels, the culmination of these results are most favorable for CpG ODN 2395, although Poly(I:C) appears more immunostimulatory.

Canine qPCR was unable to provide clear differences between TLR mRNA between immune and OS cell lines, unlike what we observed in our murine studies. While this does indicate TLR mRNA are not upregulated in canine OS, it does not provide a clear guide for agonist selection. Similar to the murine results, however, TNFα is the major immune cytokine stimulated, but IFNα levels rarely responded to TLR agonism. IL-12 levels were also unchanged in response to TLR agonism. These negative results (IFNα and IL-12) may be dose-dependent or time-dependent, as we only investigated a few concentrations of agonist and utilized a single standard incubation time. The Nike and 030-D canine immune cell lines are also poorly characterized and evaluation of canine peripheral blood mononuclear cells (PBMCs) may better represent the immune response to TLR agonism in dogs. Canine OS cell lines, however, had significant increases in both IL-6 and IL-8 for Pam3CSK4 and Poly(I:C), with less effects on VEGF, while CpG ODN 2395 had minimal pro-tumorigenic activity. This lack of pro-tumorigenic activity also shifted favoritism towards TLR9 agonism within the canine lines.

Overall, this *in vitro* data does support evaluating all 3 agonists (Pam3CSK4, Poly(I:C), and CpG ODN 2395) in a syngeneic murine model of OS, as there is no clear winner from the expression and functionality studies investigated. While this data suggests CpG ODN 2395 may be least pro-tumorigenic, we are fully aware that these experiments do not adequately capture the many interactions that occur within a living organism undergoing cancer therapy. Thus, application of these agonists within a murine model of OS is necessary for full appreciation of each agonist's capacity to be an OS immunotherapeutic.

FIGURES AND TABLES

TLR Agonist	Origin	Ligand for	InvivoGen#
Pam3CSK4	Synthetic triacylated bacterial lipoprotein	TLR1/2	tlrl-pms
LTA-SA Purified	Lipotechoic acid from *S. aureus*	TLR2	tlrl-pslta
Poly(A:U)	Synthetic dsRNA	TLR3	tlrl-pau
Poly(I:C) HMW	High molecular weight synthetic dsRNA	TLR3	tlrl-pic
LPS-B5 Ultrapure	LPS from *E. coli* 055:B5; enzymatically treated to remove lipopeptides	TLR4	tlrl-pb5lps
Imiquimod (R837)	Synthetic imidazoquinoline amine	TLR7	tlrl-imq
E. coli ssDNA plus LyoVec	Lyophilized *E. coli* K12 ssDNA combined with LyoVec lipid-based transfection reagent	TLR9	tlrl-ssec
CpG ODN 2395	Synthetic Class C CpG oligonucleotide; human/murine specific	TLR9	tlrl-2395

Table 2.1: Summary of TLR agonists investigated for potential OS immunotherapy. The TLR agonists listed were initially screened for stimulatory activity in immune and OS cell lines to assess if certain agonists had divergent activity between immune and OS cells.

Murine TaqMan Gene Expression Assay	Gene Name	Amplicon Length (bp)
Mm00442346_m1	TLR2	69
Mm01207404_m1	TLR3	121
Mm00445273_m1	TLR4	87
Mm00446590_m1	TLR7	125
Mm00446193_m1	TLR9	60
Mm99999915_g1*	GAPDH	107
Mm01197698_m1	GUSB	71
Mm01143545_m1	HMBS	81
Mm02619580_g1*	ACTB	143
Mm00437762_m1	B2M	77

Table 2.2: Murine genes investigated and the primer/probe sets utilized for RT-PCR.
*Designates an assay that may detect genomic DNA.

Canine TaqMan Gene Expression Assay	Gene Name	Amplicon Length (bp)
Cf03034088_s1**	TLR2	110
AJWR2PO***	TLR3	62
Cf02622203_g1*	TLR4	120
Cf02710573_s1**	TLR7	124
Cf02622347_g1*	TLR9	87
Cf02717353_g1*	TLR9	136
Cf04419463_gh*	GAPDH	54
Cf02622808_m1	GUSB	75
Cf02626258_m1	HPRT1	129
Cf02681523_g1	RPS18	160

Table 2.3: Canine genes investigated and the primer/probe sets utilized for RT-PCR.
*Designates an assay that may detect genomic DNA, **designates an assay that will detect genomic DNA, and ***designates a custom-designed assay.

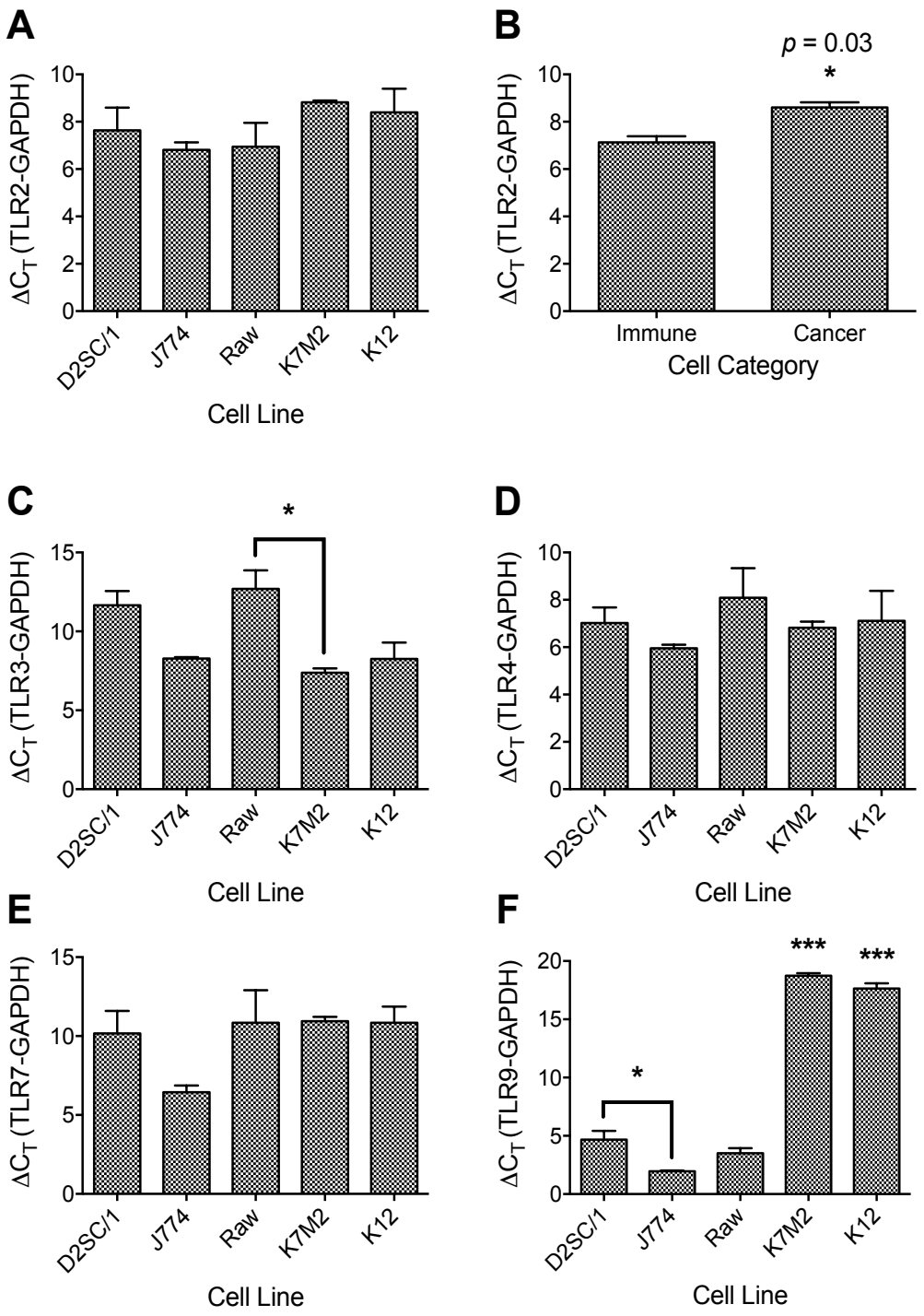

Figure 2.1: Normalized qPCR mRNA levels for TLRs 2, 3, 4, 7, and 9 in murine immune and OS cell lines (A-F). Immune cells include D2SC/1, J774, and RAW. OS cells include K7M2 and K12. Murine OS cells as a group exhibit less expression of TLR2 compared to immune cells **(B)**. No amplification for TLR9 **(F)** was observed for either OS line; therefore, the C_T level was arbitrarily set at 41 for graphical purposes. K7M2 and K12 are also statistically different from all immune cell lines for TLR9. One-way ANOVA. *p<0.05 and ***p<0.001

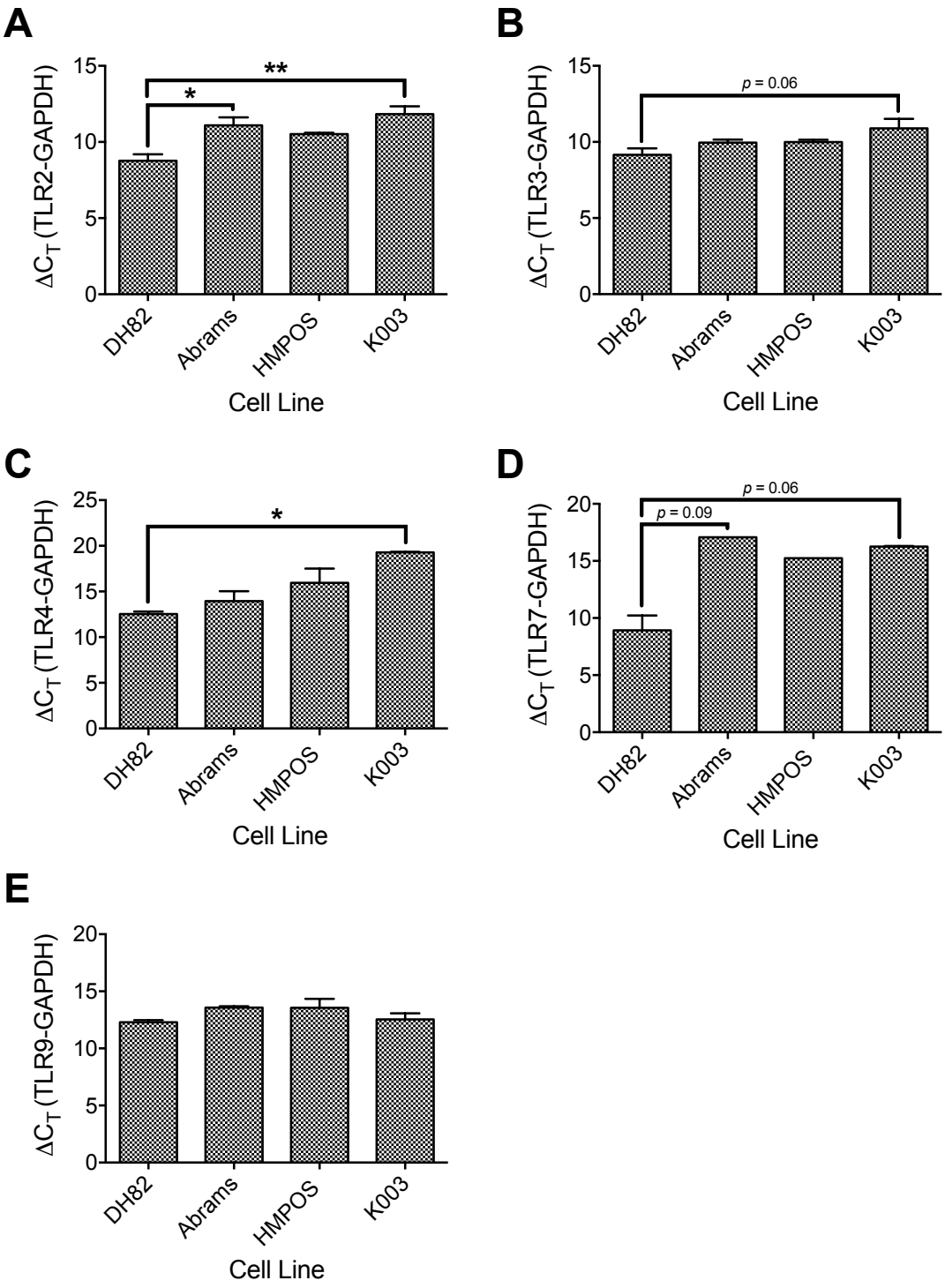

Figure 2.2: Normalized qPCR mRNA levels for TLRs 2, 3, 4, 7, and 9 in canine immune and OS cell lines (A-E). Immune cells are represented by DH82. OS cells include Abrams, HMPOS, and K003. TLR2 **(A)** and TLR4 **(B)** have lower expression in Abrams and/or K003 OS cell lines compared to DH82. Expression of TLR3 **(C)**, TLR7 **(D)**, and TLR9 **(E)** are not significantly different between canine immune and OS cells. One-way ANOVA. *$p<0.05$ and **$p<0.01$

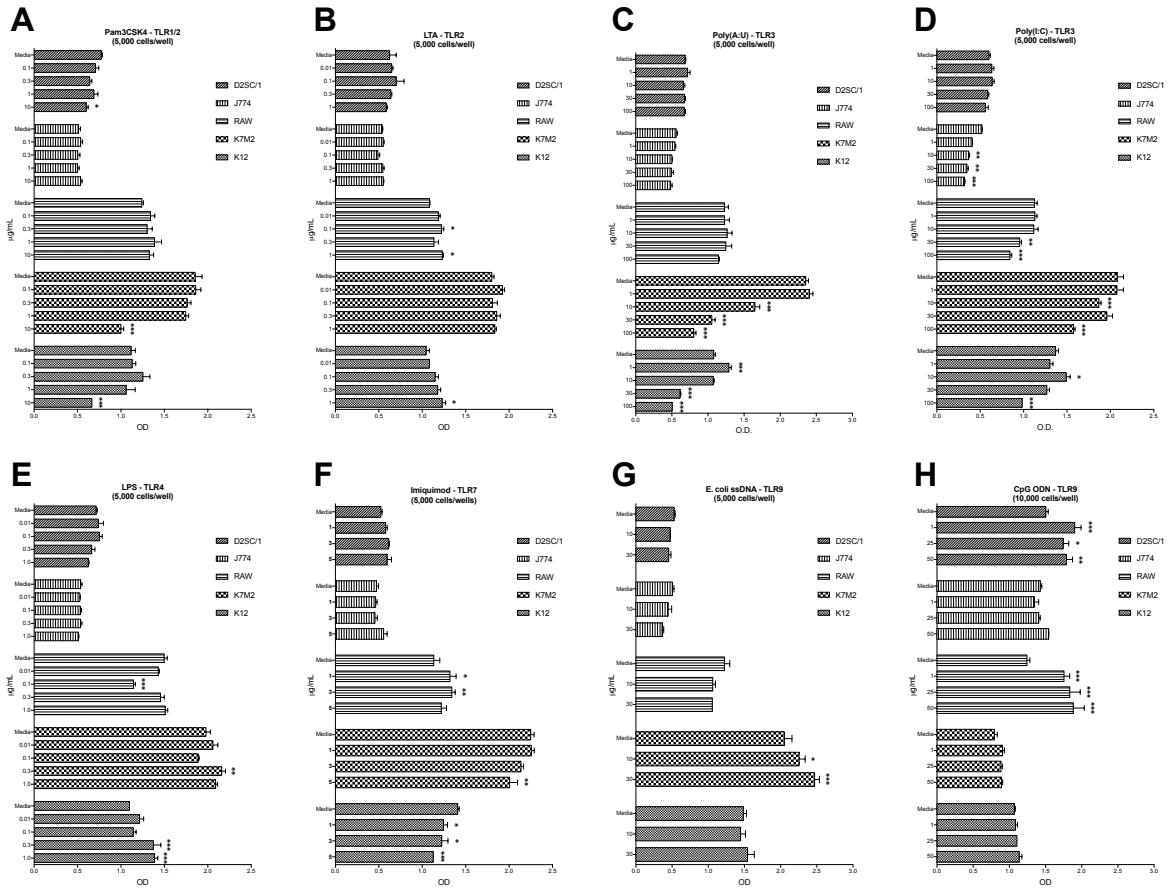

Figure 2.3: Cellular viability as measured by MTS assay in response to various TLR agonists for murine immune and OS cell lines.
Murine immune cells represented by D2SC/1, J774, and RAW. Murine OS cells represented by K7M2 and K12 (as viewed from top to bottom of each graph). Note how Pam3CSK4 **(A)**, Poly(A:U) **(C)**, Poly(I:C) **(D)**, and Imiquimod **(F)** treatments cause decreased cellular viability within the OS cell lines. CpG ODN 2395 **(H)** treatment does not affect cellular viability within the OS cells. Two-way ANOVA. *p<0.05, **p<0.01, and ***p<0.001

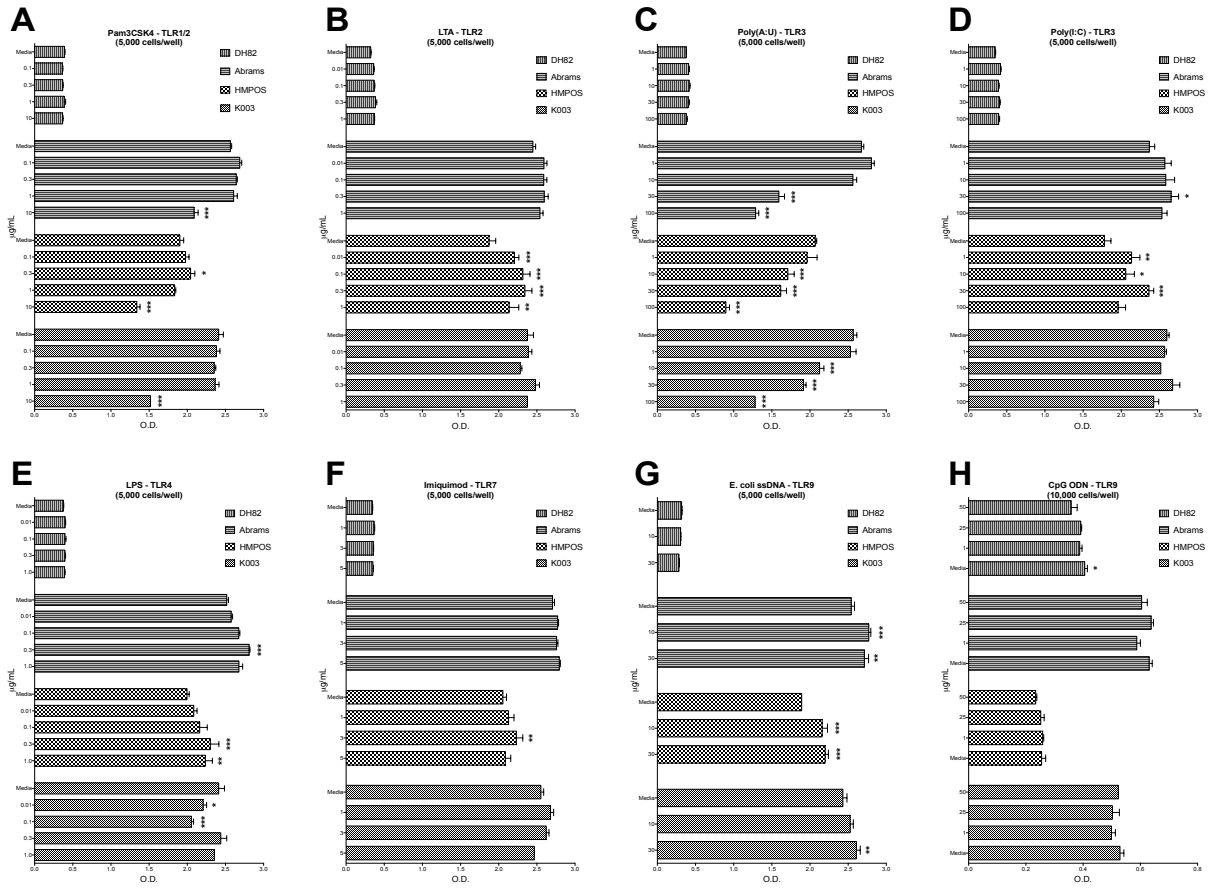

Figure 2.4: Cellular viability as measured by MTS assay in response to various TLR agonists for canine immune and OS cell lines.
Canine immune cells represented by DH82. Canine OS cells represented by Abrams, HMPOS, and K003 (as viewed from top to bottom of each graph). Note how Pam3CSK4 **(A)** and Poly(A:U) **(C)** treatments cause decreased cellular viability within the OS cell lines. CpG ODN 2395 **(H)** treatment does not affect cellular viability within the OS cells. Two-way ANOVA. *p<0.05, **p<0.01, and ***p<0.001

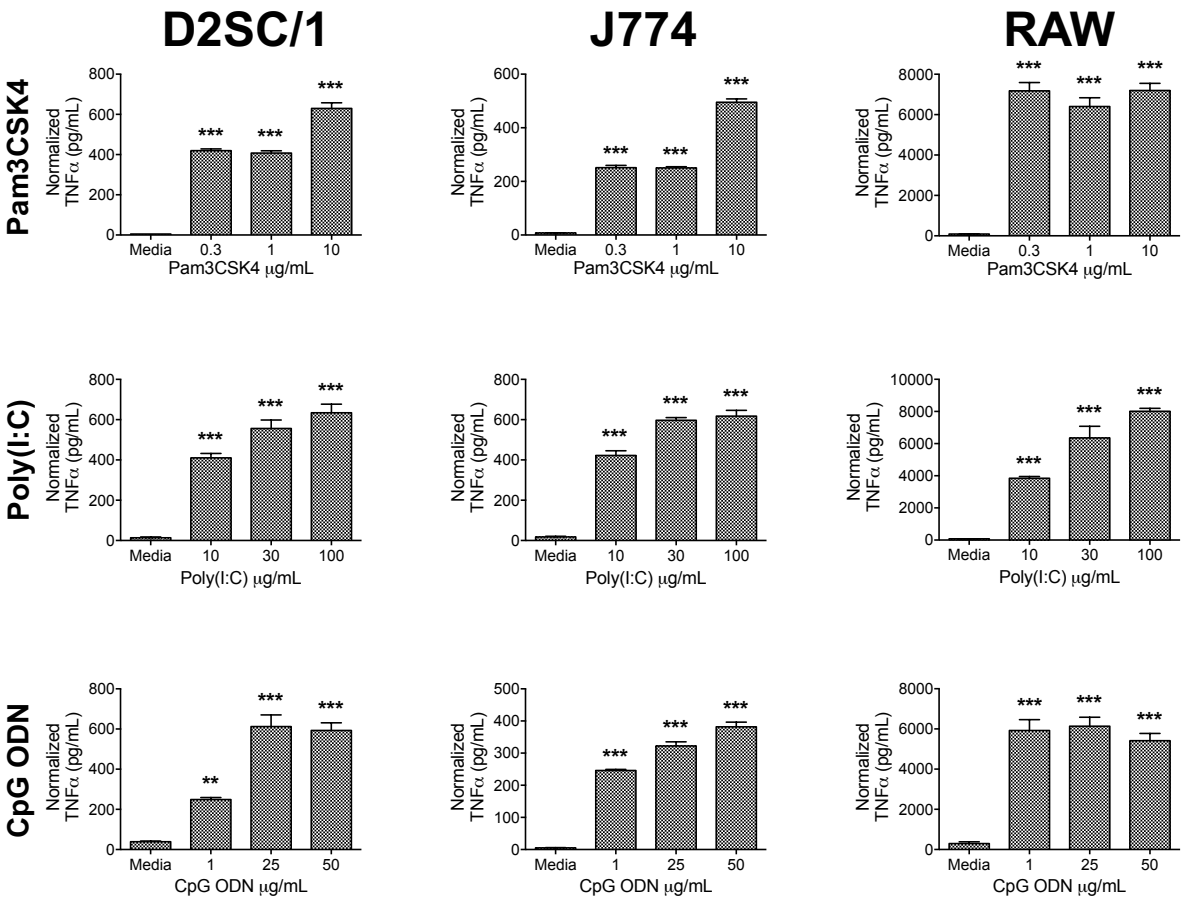

Figure 2.5: Normalized TNFα secretion in response to Pam3CSK4, Poly(I:C), or CpG ODN 2395 treatment in murine immune cells.
All three murine immune cell lines investigated (D2SC/1, J774, and RAW) exhibit robust secretion of TNFα in response to TLR agonist treatment. This secretion is often dose-dependent. One-way ANOVA. $^{**}p<0.01$ and $^{***}p<0.001$

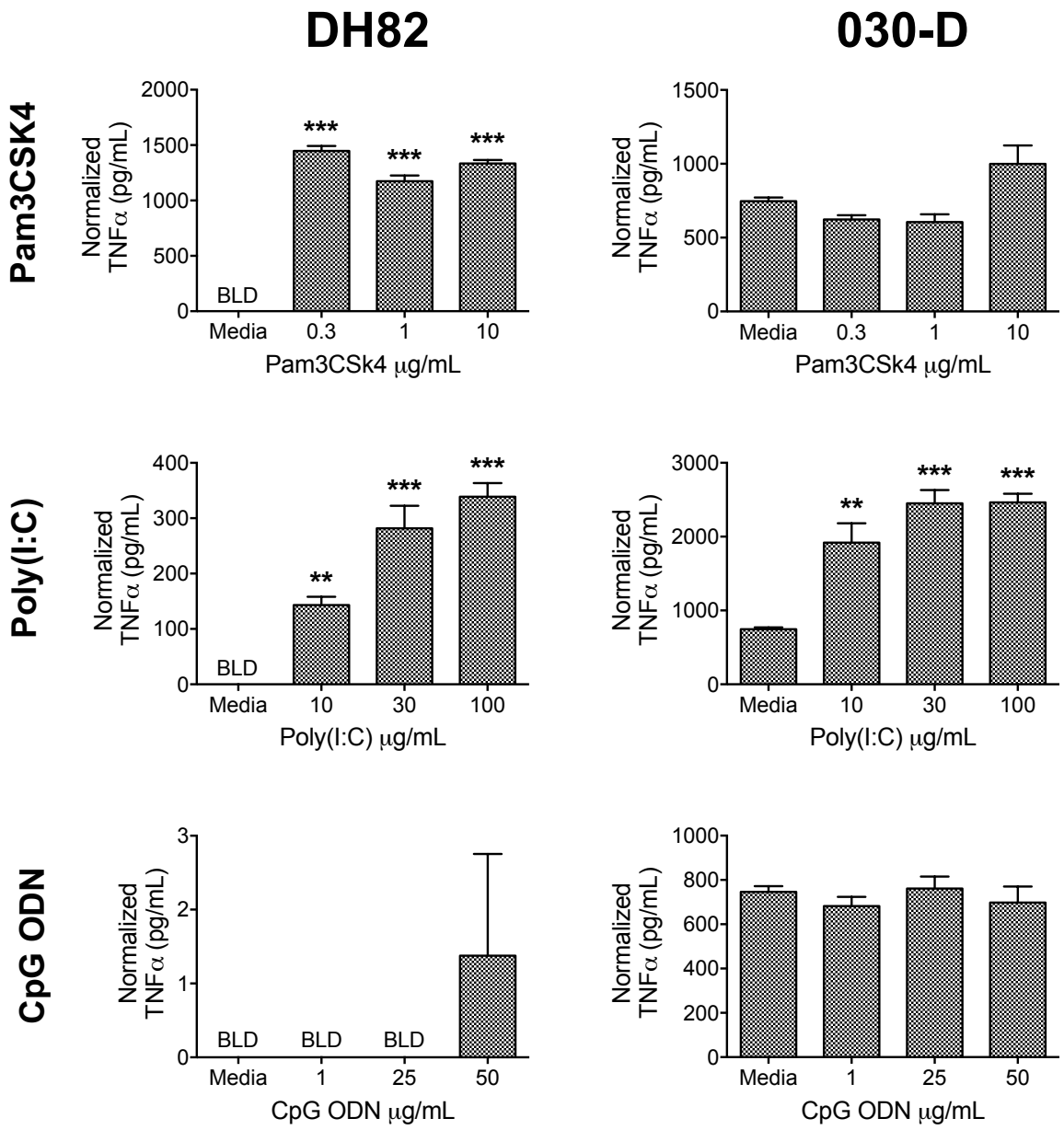

Figure 2.6: Normalized TNFα secretion in response to Pam3CSK4, Poly(I:C), or CpG ODN 2395 treatment in canine immune cells.
DH82 responds to all three agonists with increases in TNFα secretion, whereas 030-D only increases secretion in response to Poly(I:C). The Nike cell line did not secrete measurable levels of TNFα, regardless of treatment or concentration. One-way ANOVA. $^{**}p<0.01$ and $^{***}p<0.001$

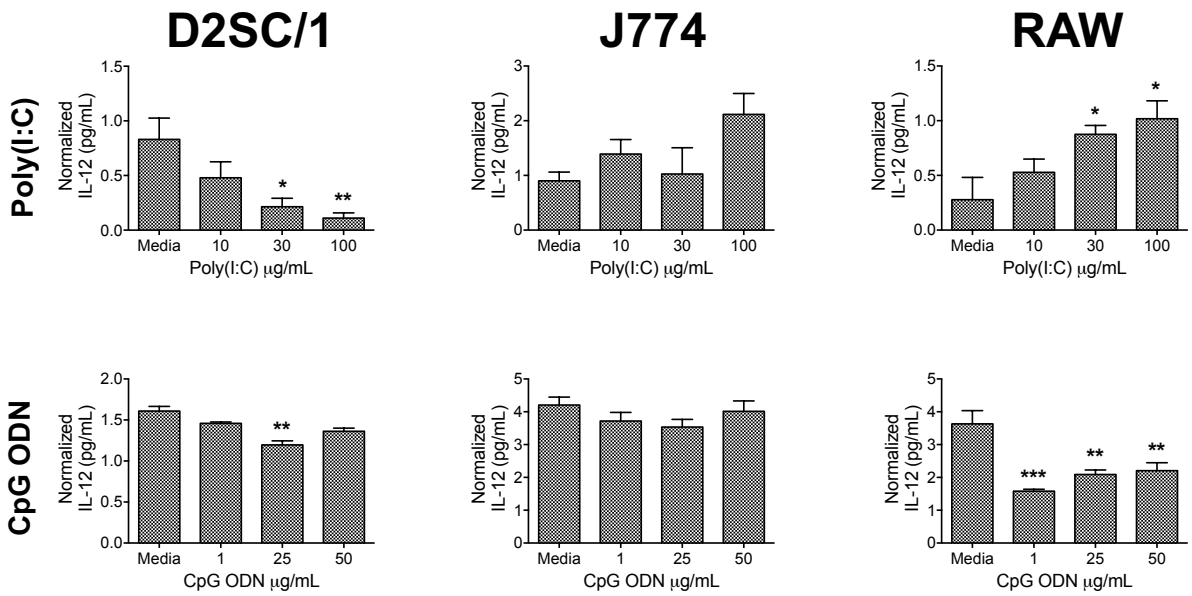

Figure 2.7: Normalized IL-12 secretion in response to Pam3CSK4, Poly(I:C), or CpG ODN 2395 treatment in murine immune cells.
Poly(I:C) was the only agonist to successfully increase IL-12 secretion, which was limited to the RAW cell line. No measurable secretion of IL-12 was observed with Pam3CSK4 treatment (data not shown). One-way ANOVA. *p<0.05, **p<0.01, and ***p<0.001

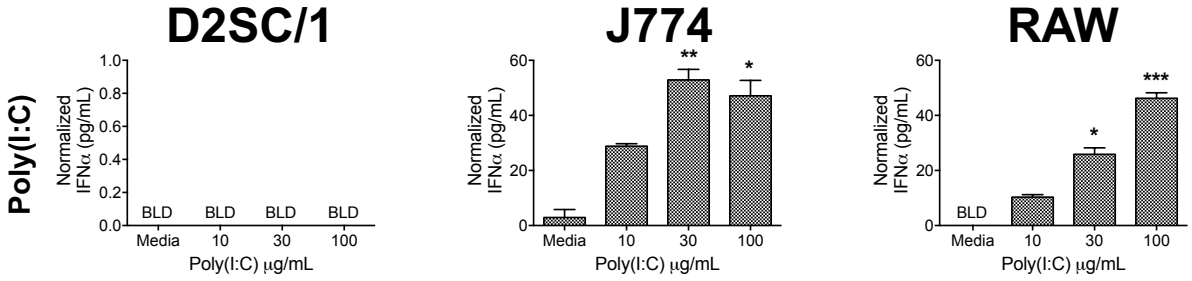

Figure 2.8: Normalized IFNα secretion in response to Pam3CSK4, Poly(I:C), or CpG ODN 2395 treatment in murine immune cells.
Poly(I:C) was the only agonist to successfully increase IFNα secretion and was limited to J774 and RAW cells. No measurable secretion of IFNα was observed with Pam3CSK4 or CpG ODN 2395 treatment (data not shown). One-way ANOVA. *p<0.05, **p<0.01, and ***p<0.001

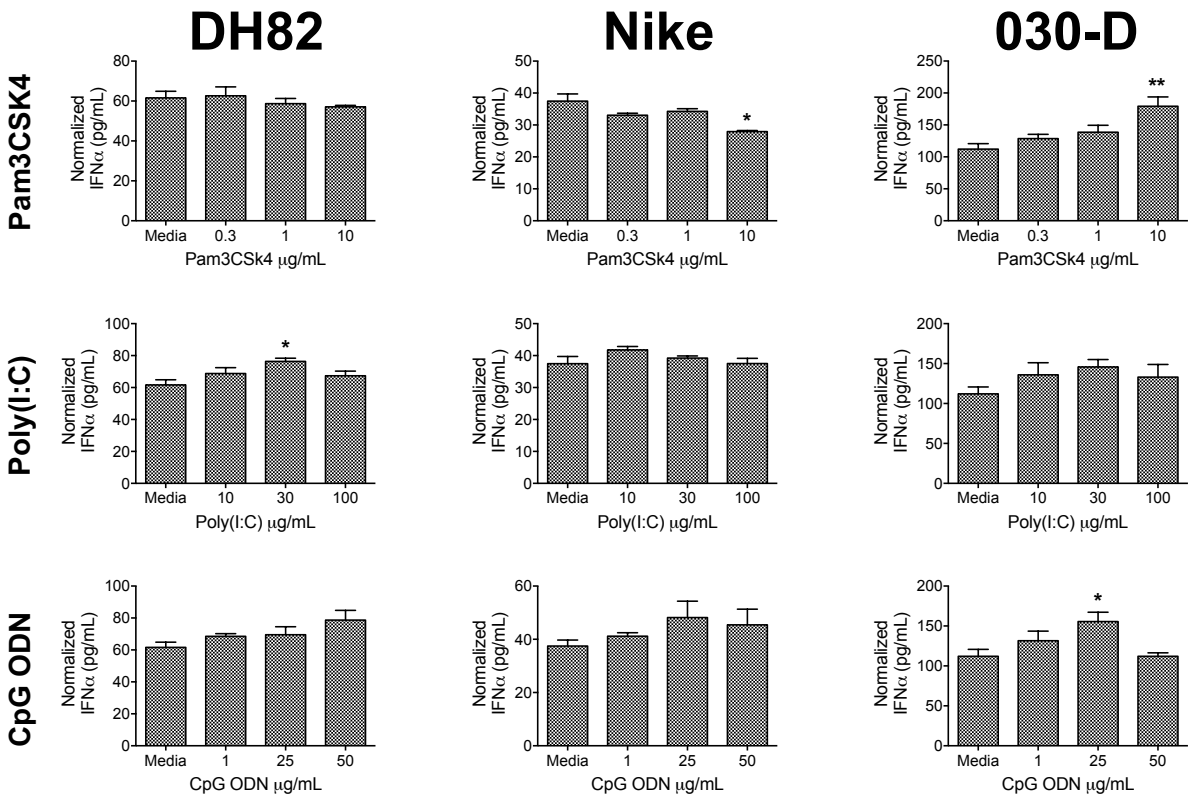

Figure 2.9: Normalized IFNα secretion in response to Pam3CSK4, Poly(I:C), or CpG ODN 2395 treatment in canine immune cells.

All three agonists induce minimal to no significant increases in IFNα amongst canine immune cell lines. One-way ANOVA. *$p<0.05$ and **$p<0.01$

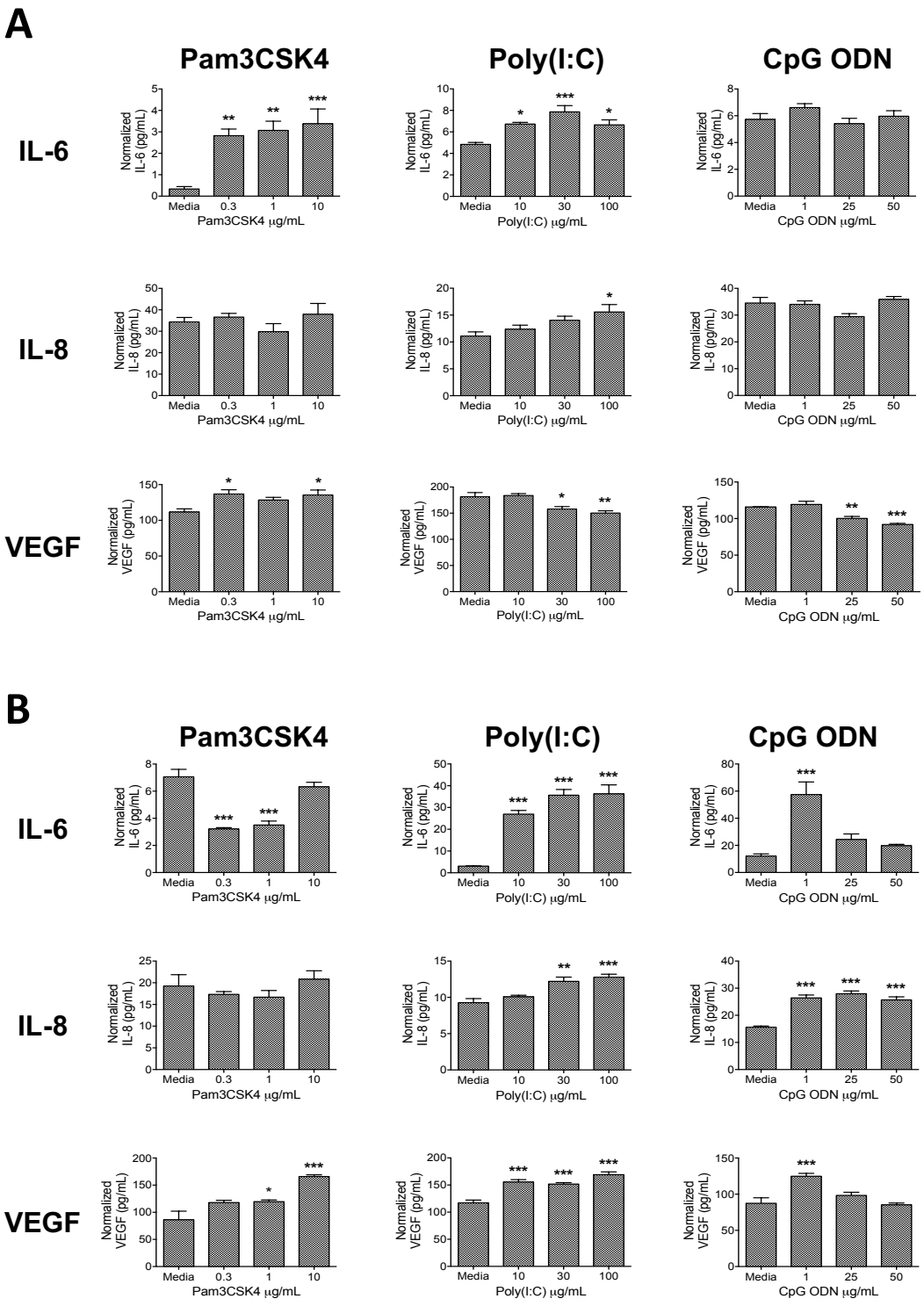

Figure 2.10: Normalized pro-tumorigenic cytokine secretion in response to Pam3CSK4, Poly(I:C), and CpG ODN 2395 in murine OS cells.
K7M2 **(A)** and K12 **(B)** both exhibit variable responses in IL-6, IL-8, and VEGF secretion. However, CpG ODN 2395 has the most consistent response across both cell lines, in regard to minimizing overall upregulation of each cytokine. One-way ANOVA. *p<0.05, **p<0.01, and ***p<0.001

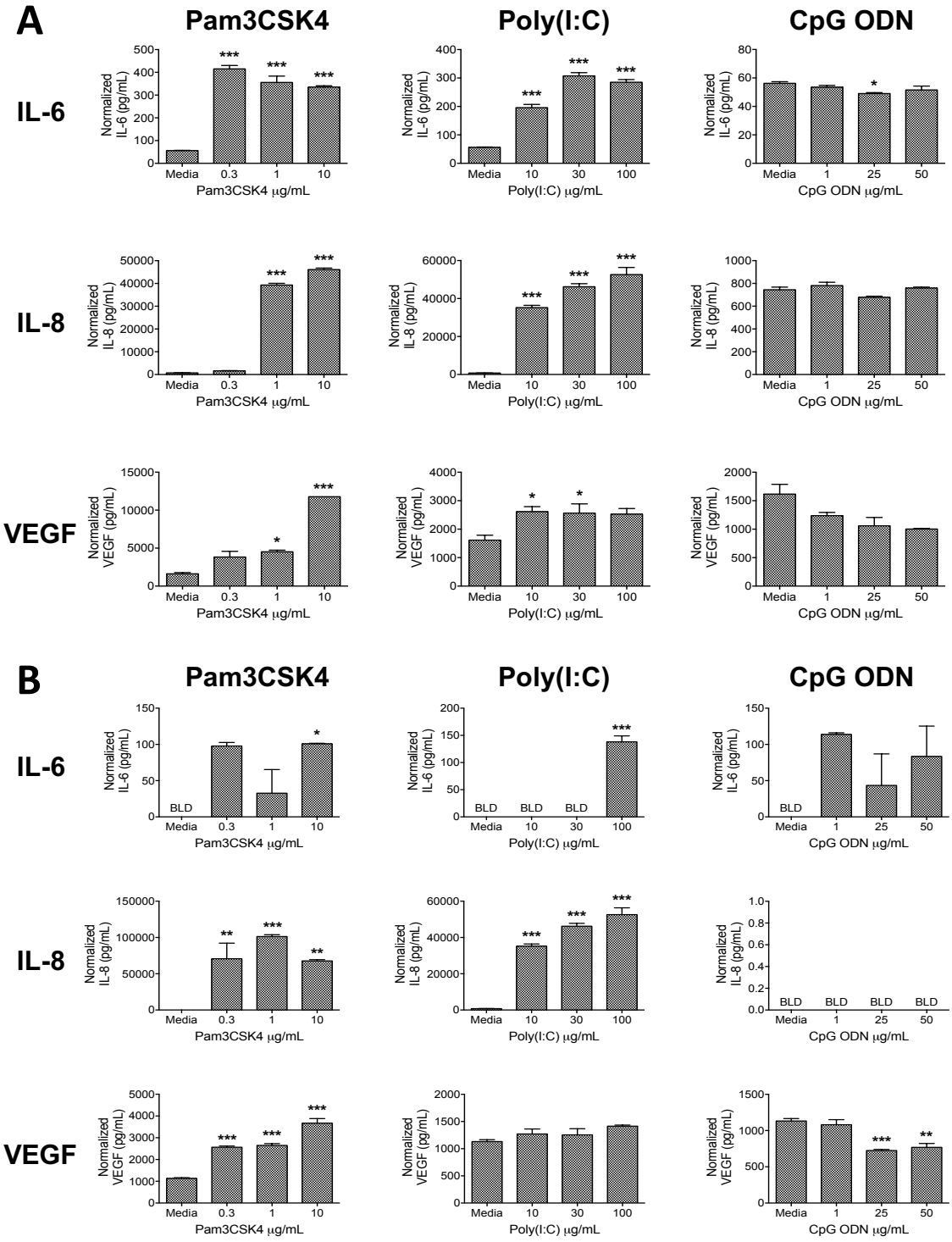

Figure 2.11A-B: Normalized pro-tumorigenic cytokine secretion in response to Pam3CSK4, Poly(I:C), and CpG ODN 2395 in canine Abrams and HMPOS OS cells. Abrams **(A)** and HMPOS **(B)** exhibit variable responses in IL-6, IL-8, and VEGF secretion. Similar to the murine OS cell lines, CpG ODN 2395 has the most consistent response across both cell lines, in regard to minimizing overall upregulation of each cytokine. One-way ANOVA. *p<0.05, **p<0.01, and ***p<0.001

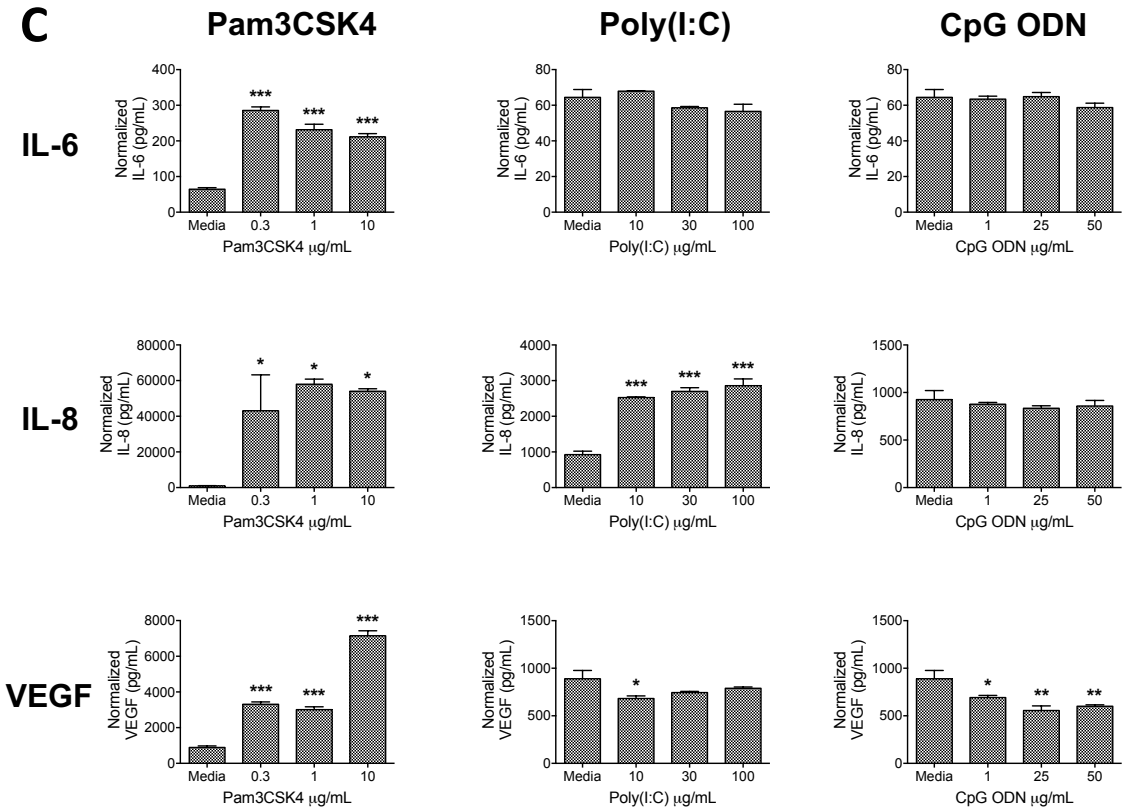

Figure 2.11C (cont.): Normalized pro-tumorigenic cytokine secretion in response to Pam3CSK4, Poly(I:C), and CpG ODN 2395 in canine K003 OS cells.
K003 **(C)** also exhibits variable responses in IL-6, IL-8, and VEGF secretion. While Poly(I:C) does not result in increased IL-6 or VEGF secretion, CpG ODN 2395 does not increase secretion of any pro-tumorigenic cytokine investigated (IL-6, IL-8, and VEGF). These findings corroborate what is observed in Abrams and HMPOS, along with the murine OS cell lines K7M2 and K12. One-way ANOVA. *p<0.05, **p<0.01, and ***p<0.001

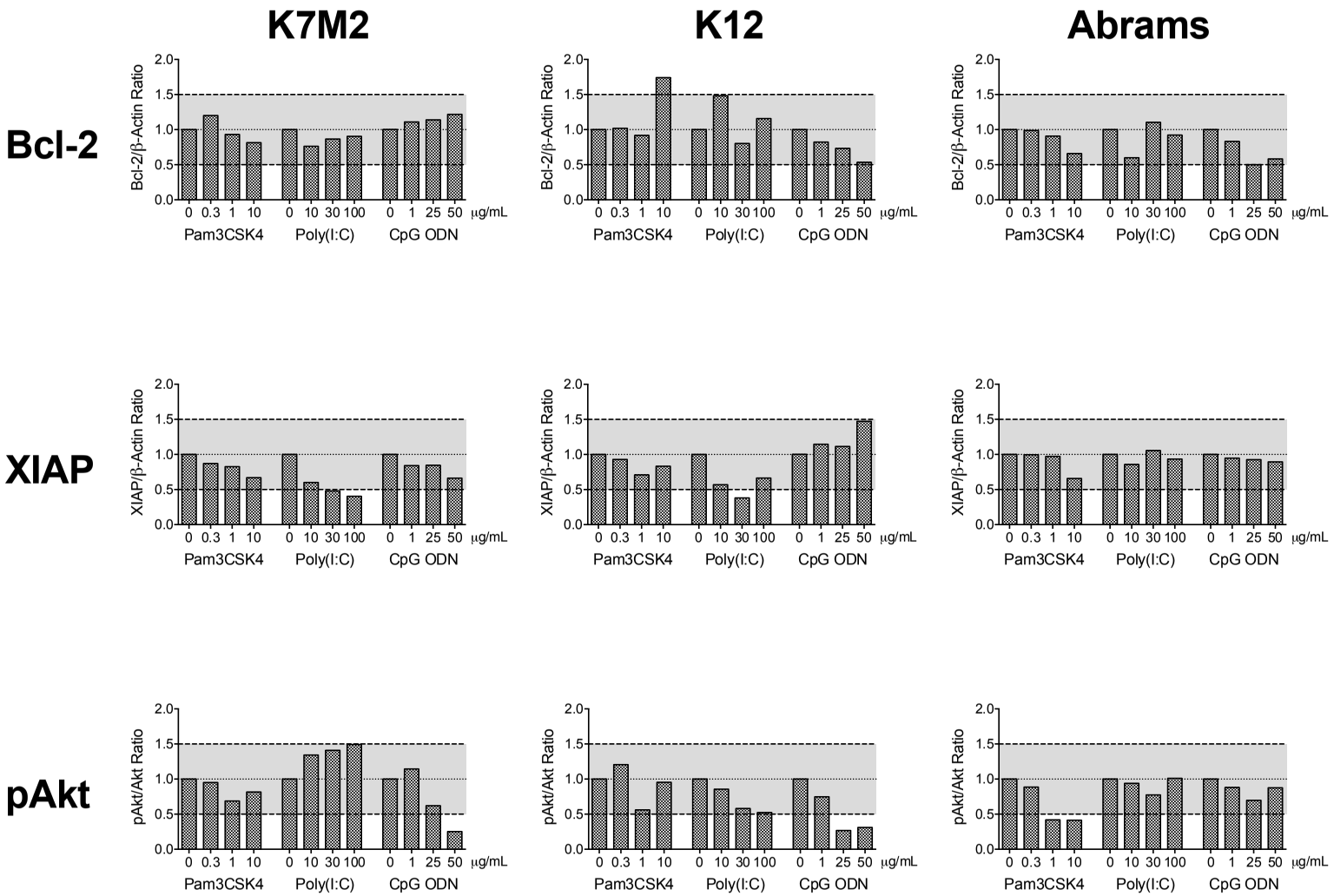

Figure 2.12: Pro-tumorigenic protein expression via Western Blot in response to Pam3CSK4, Poly(I:C), and CpG ODN 2395 for murine (K7M2, K12) and canine (Abrams) OS cell lines.
Bcl-2, XIAP, and pAkt are normalized relative to β-actin or Akt. Data is expressed as the percent qualitative change compared to untreated controls (control=1); the shaded area represents ±50% change. No TLR agonist creates consistent upregulation or downregulation of any protein over 50% across all cell lines investigated.

LITERATURE CITED

1. Kawasaki T, Kawai T. Toll-like receptor signaling pathways. Front Immunol 2014;5:461.

2. Botos I, Segal DM, Davies DR. The structural biology of toll-like receptors. Structure 2011;19:447-459.

3. Hua Z, Hou B. TLR signaling in B-cell development and activation. Cell Mol Immunol 2013;10:103-106.

4. Gelman AE, Zhang J, Choi Y, et al. Toll-like receptor ligands directly promote activated $CD4^+$ T cell survival. J Immunol 2004;172:6065-6073.

5. Rahman AH, Taylor DK, Turka LA. The contribution of direct TLR signaling to T cell response. Immunol Res 2009;45:25-36.

6. Shi M, Chen X, Ye K, et al. Application potential of toll-like receptors in cancer immunotherapy. Medicine (Baltimore) 2016;95:e3951.

7. Young E, Ouchi T. The application of toll like receptors for cancer therapy. Int J Biol Sci 2010;6:675-681.

8. Jouhi L, Renkonen S, Atula T, et al. Different toll-like receptor expression patterns in progression towards cancer. Front Immunol 2014;5:638.

9. Takeda K, Akira S. Toll-like receptors in innate immunity. Int Immunol 2005;17:1-14.

10. Allenspach K, House A, Smith K, et al. Evaluation of mucosal bacteria and histopathology, clinical disease, activity and expression of toll-like receptors in German Shepherd dogs with chronic enteropathies. Vet Microbiol 2010;146:326-335.

11. Bazzocchi C, Mortarino M, Comazzi S, et al. Expression and function of toll-like receptor 2 in canine blood phagocytes. Vet Immunol Immunopathol 2005;104:15-19.

12. Boozer LB, Davis TW, Borst LB, et al. Characterization of immune cell infiltration into canine intracranial meningiomas. Vet Pathol 2012;49:784-795.

13. Silva E, S Leitão, Henriques S, et al. Gene transcription of TLR2, TLR3, LPS ligands and prostaglandin synthesis enzymes are up-regulated in canine uteri with cystic endometrial hyperplasia-pyometra complex. J Reprod Immunol 2010;84:66-74.

14. Kawarai S, Sato K, Horiguchi A, et al. Potential immunological adjuvant of 'K'-type CpG-oligodeoxynucleotides enhanced the cell proliferation and IL-6 mRNA transcription in canine B cells. J Vet Med Sci 2011;73:177-184.

15. Bonnefont-Rebeix C, Marchal T, Bernaud J, et al. Toll-like receptor 3 (TLR3): a new marker of canine monocyte-derived dendritic cells (cMo-DC). Vet Immunol Immunopath 2007;118:134-139.

16. Palm NW, Medzhitov R. Pattern recognition receptors and control of adaptive immunity. Immunol Rev 2009;227:221-233.

17. Ozato K, Tsujimura H, Tamura T. Toll-like receptor signaling and regulation of cytokine gene expression in the immune system. BioTechniques 2002;33:S66-S75.

18. Medzhitov R, Janeway C Jr. Innate immune recognition: mechanisms and pathways. Immunol Rev 2000;173:89-97.

19. Adib-Conquy M, Scott-Algara D, Cavaillon JM, et al. TLR-mediated activation of NK cells and their role in bacterial/viral immune responses in mammals. Immunol Cell Biol 2014;92:256-262.

20. Basith S, Manavalan B, Yoo TH, et al. Roles of toll-like receptors in cancer: a double-edged sword for defense and offense. Acta Pharm Res 2012;35:1297-1316.

21. Khanna C, Prehn J, Yeung C, et al. An orthotopic model of murine osteosarcoma with clonally related variants differing in pulmonary metastatic potential. Clin Exp Metastasis 2000;18:261-271.

22. Andersen CL, Jensen JL, Ørntoft TF. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res 2004;64:5245-5250.

23. Adepoju LJ, Geiger JD. Antitumor activity of polyuridylic acid in human soft tissue and bone sarcomas. J Surg Res 2010;164:104-114.

24. Shatz M, Menendez D, Resnick MA. The human TLR innate immune gene family is differentially influenced by DNA stress and p53 status in cancer cells. Cancer Res 2010;72:3948-3957.

25. Moriya R, Takahashi K, Kitahara A, et al. Possible involvement of PI3K-dependent pathways in the increased VEGF120 release from osteoblastic cells preloaded with palmitate in vitro. Biochem Biophys Res Commun 2014;445:275-281.

26. Jing Y, Liang H, Zhang Y, et al. Up-regulation of toll-like receptor 9 in osteosarcoma. Anticancer Res 2015;35:5839-43.

27. Dovedi SJ, Adlard AL, Ota Y, et al. Intravenous administration of the selective toll-like receptor 7 agonist DSR-29133 leads to anti-tumor efficacy in murine solid tumor models which

can be potentiated by combination with fractionated radiotherapy. Oncotarget 2016;7:17035-17046.

28. Broadhead ML, Clark JCM, Myers DE, et al. The molecular pathogenesis of osteosarcoma: a review. Sarcoma 2011;2011;Article ID 959248.

CHAPTER III

INVESTIGATING THE PRO- AND ANTI-TUMORIGENIC ACTIVITY OF SELECT TOLL-LIKE RECEPTOR AGONISTS IN A MURINE MODEL OF OSTEOSARCOMA

Abstract

The K7M2 syngeneic Balb/c model of metastatic OS is a popular model for the pre-clinical evaluation of new OS therapeutics and for studying the mechanisms underlying OS pathogenesis. This model has several variations, including an orthotopic and tail vein model, with the tail vein model producing fast but consistent replication of experimental OS pulmonary metastasis. Although mouse models of disease do not always accurately recapitulate or predict outcomes in large animal models of disease, it was prudent to evaluate and characterize the activity of these potential therapeutics in a small animal model prior to employing them within a clinical setting. Based upon our findings in **Chapter II**, we choose to evaluate Pam3CSK4, Poly(I:C), and CpG ODN 2395 in this clinically-relevant murine model of OS to further delineate their immunotherapeutic potential before use in dogs with spontaneous OS.

Our findings indicate Poly(I:C) and CpG ODN 2395 both inhibit the development of pulmonary metastasis in this model, while Pam3CSK4 appears to have tumor-promoting capabilities. However, the effect of Poly(I:C) is apparently short-lived while CpG ODN 2395 has marked long-term positive outcomes, including significantly increased survival. We also show CpG ODN 2395 promotes an increase in T_h1 and cytotoxic T cells, but does not increase antigen-specific cytotoxicity towards K7M2 nor does its therapeutic effect appear to be due to macrophage-associated killing. Additionally, we uncover the ability of CpG ODN 2395 to inhibit pulmonary

metastasis in a treatment/tumor emboli scheme closely mimicking the clinical course of OS therapy and disease in dogs.

Introduction

The K7M2 OS cell line is derived from a spontaneous Balb/c OS. It was created through intraosseous injection of the parent cell line K7 with reimplantation of subsequent K7 lung metastases into a parosteal tibial muscle flap. This procedure was repeated until the K7M2 cell line was developed. The K7M2 model can be used in 2 fashions: 1) as a syngeneic orthotopic model with eventual spontaneous metastasis or 2) tail vein injection for accelerated creation of experimental pulmonary metastases. With the tail vein injection method, death from pulmonary metastases is 100% with a median survival time (MST) of 17 days, indicating its aggressive course but also allowing for the efficient study of OS metastasis in a syngeneic model.[1,2] The K7M2 cell line also expresses some classical proteins of bone, such as osteopontin and osteocalcin, but lacks expression of alkaline phosphatase.[1,3]

Using the K7M2 tail vein injection model, we sought to evaluate whether our 3 selected TLR agonists from **Chapter II** (Pam3CSK4, Poly(I:C), and CpG ODN 2395) would be effective in reducing pulmonary metastasis and increasing survival time. We also studied how these TLR agonists shape the immunophenotype of lymphocytes within OS-bearing mice and if TLR agonism increased specific cell-mediated cytotoxicity against K7M2. We hypothesized that TLR agonism in this model would produce variable results, e.g. some agonists may promote a pro-tumorigenic phenotype while others may promote an anti-tumorigenic phenotype. The results from this chapter indicate OS pulmonary metastasis in the K7M2 tail vein injection model can be inhibited by CpG ODN 2395, that CpG ODN 2395 induces increased numbers of anti-tumor lymphocyte

subpopulations in comparison to Pam3CSK4 and Poly(I:C), but that this reduction in lung tumor metastasis is likely not due to increases in antigen-specific cell-mediated cytotoxicity. We also show that while CpG ODN 2395 induces phagocytosis in murine immune cells, macrophages do not appear to be the single critical component in the reduction of metastasis mediated by CpG ODN 2395 therapy. CpG ODN 2395 also appears to have a narrow window of efficacy in the prevention of lung metastasis development, which requires further evaluation in future studies, but based off the significant increases in survival time that it can produce in this murine OS model, it is still an attractive therapy for use in dogs with spontaneous OS.

Material and Methods

Cell Lines

The murine OS cell line K7M2, murine immune cell lines J774 and RAW, and the canine immune cell lines DH82 and Nike were cultured in DMEM supplemented with 10% FBS and 1% P/S at 37ºC and 5% CO_2 in a humidified incubator. Prior to expansion of the K7M2 cell line for *in vivo* experiments, representative cell culture media aliquots were confirmed negative for *Mycoplasma* spp. via PCR performed at the University of Illinois Veterinary Diagnostic Laboratory.

Mice

Six- to eight-week-old female Balb/c mice were purchased from Charles River Laboratories (Wilmington, MA). All mice were housed within the University of Illinois Division of Animal Resources facilities. All studies were approved by the University of Illinois Institutional Animal Care and Use Committee (IACUC).

Prophylactic Toll-Like Receptor Agonist Treatment

Mice were injected subcutaneously (SQ) over the scruff of the neck weekly for four weeks with either Pam3CSK4 (10 μg/mouse), Poly(I:C) (100 μg/mouse), or CpG ODN (50 μg/mouse) in Hank's Balanced Salt Solution (HBSS). A subset of mice were injected with a tumor cell lysate consisting of $1x10^6$ mycoplasma-negative K7M2 cells in addition to their respective TLR agonist treatment. The tumor cell lysate was prepared by subjecting tumor cells to 8-10, -80°C freeze/thaw cycles followed by confirmation of <5% cell viability by trypan blue exclusion. Total injection volume per mouse was 250 μL, regardless of treatment type (TLR agonist only vs. TLR agonist plus tumor cell lysate). Sham controls were injected with an equal volume of HBSS. Three days after the 4^{th} TLR agonist treatment, mice were injected with $1x10^6$ mycoplasma-negative K7M2 in 200 μL HBSS via tail vein (day 0). Two, three, or four additional SQ TLR injections (+/- K7M2 tumor cell lysate) were continued on a weekly basis. Mice were then followed for survival time or sacrificed at day 14, day 21, or day 28 for assessment of lung tumor burden, collection of serum for cytokine assessment, and collection of spleens for flow cytometry.

Flow Cytometry

Spleens were harvested from mice under the prophylactic TLR agonist treatment scheme (without concurrent tumor cell lysate treatment) at day 14 or day 28 post-K7M2 tail vein injection. Spleen weights were obtained, then spleens were homogenized between the frosted edges of two glass slides and mononuclear cells collected via gradient centrifugation using Histopaque 1.083 (Sigma-Aldrich, St. Louis, MO) according to manufacturer's instructions. Total mononuclear cell counts were determined via hemocytometer chamber counts for each sample.

Flow cytometry was performed on BD Accuri C6 or BD LSR II flow cytometry analyzers (BD Biosciences, San Jose, CA) using triple-stained samples to assess the following lymphocyte subpopulations: cytotoxic T lymphocytes (CTL), T_h1-polarized lymphocytes (T_h1), T regulatory lymphocytes (T_{reg}), and natural killer cells (NK). Antibody-fluorochrome conjugates for each lymphocyte population are listed in **Table 3.1**. Fluorescence Minus One (FMO), compensation, and isotype controls were run concurrently. All antibodies and flow reagents were manufactured by eBioscience (Thermo Scientific).

Briefly, washed splenocytes were resuspended in chilled Flow Cytometry Staining Buffer with F_c Block (anti-mouse CD16/32; 0.5 μg/10^6 cells) and incubated for 15 minutes at 4ºC. Following blocking, splenocytes were washed in chilled flow buffer and then split into separate samples for triple-staining of lymphocyte subpopulations (**Table 3.1**). Staining for each antibody-fluorochrome conjugate was performed at ≤1.0 μg/10^6 cells. Samples were incubated in the dark for 30 minutes, then washed 1x in chilled flow buffer followed by resuspension in chilled Fixation/Permeabilization Buffer. Samples were then stored overnight in the dark at 4ºC. The following morning, all samples were resuspended in chilled Permeabilization Buffer and samples for T_{reg} analysis were incubated with anti-mouse FoxP3-APC antibody for 30 minutes in the dark at 4ºC. Following washing of T_{reg} samples, all samples were pelleted for removal of Permabilization Buffer supernatants and resuspended in 100 μL chilled flow buffer for subsequent analysis.

Ex vivo cytotoxicity assays

Splenocytes were harvested from mice under the prophylactic TLR agonist treatment scheme using aseptic technique. Splenocytes were collected using gentle dissociation from the

capsule using curved hemostats and mononuclear cells were collected using Histopaque 1.083. Total mononuclear cell counts were determined via hemocytometer chamber counts for each sample.

Lymphocytes were plated over mitomycin c-treated K7M2 cells in 24 well plates at a lymphocyte density of $5x10^6$/mL at a 2:1 ratio with mitomycin-c treated naïve splenocytes in the presence of rmIL-2 (0.25 ng/mL) in complete T cell media. After 7 days of culture, the lymphocytes were assessed for cytotoxicity against the K7M2 and Balb/3T3 fibroblast cell lines using the LDH-based CytoTox 96 Non-Radioactive Assay kit (Promega). Percent cytotoxicity was calculated using the equation: (experimental – effector spontaneous – target spontaneous)/(target maximum – target spontaneous) *100.

Accelerated CpG ODN Treatment

Mice were injected with $1x10^6$ K7M2 in 200 µL HBSS via tail vein. Following tail vein injection mice were treated with CpG ODN on either day 0 (same day as K7M2 injection, typically within 2-4 hours after injection) or day 3, 5, or 7 post-tail vein injection. CpG ODN was administered at 50 µg/mouse in 250 µL HBSS given SQ over the scruff of the neck. Sham controls were injected with equal volumes of HBSS. Mice were followed for survival or sacrificed at day 21 for assessment of lung tumor burden and collection of serum for cytokine assessment. Mice were given weekly CpG ODN injections on a continuous basis up until the point of sacrifice or until a maximum of four treatments had been accrued for survival groups.

Liposomal Clodronate for Macrophage Depletion

Liposomal clodronate (Clodrosome) was purchased from Encapsula NanoSciences (Brentwood, TN) for macrophage depletion. To assess the efficacy of depletion *in vivo*, preliminary studies were performed where mice were treated either intraperitoneally (IP) or intranasally (IN) with clodronate over a 10-day period, following which mice were sacrificed for quantitative macrophage assessment. Quantitative macrophage assessment included collection of spleens for macrophage immunohistochemistry (IHC) and bronchoalveolar lavage (BAL) collection for lung macrophage enumeration. Subsets of mice within the accelerated CpG ODN treatment schemes were also treated with clodronate, to investigate the importance of macrophages in CpG ODN's mechanism of action (MOA).

For mice receiving IP clodronate, a 2 mg loading dose was given initially, followed by 0.5 mg doses every 3 days thereafter. Mice receiving IN treatment were administered 0.3 mg clodronate via the nares weekly. Mice receiving K7M2 tail vein injection and concurrent CpG ODN treatment were initially pre-treated with clodronate (IP or IN) for a 10-day period before tumor injection and TLR agonist administration commenced. Clodronate treatments were continued as described above for preliminary depletion studies and CpG ODN was administered weekly until day 21 when mice were sacrificed for tumor burden assessment.

Bronchoalveolar Lavage Analysis

BALs were processed by standard procedures at the University of Illinois Veterinary Diagnostic Laboratory, to obtain an automated total white blood cell count (cells/μL) and a differential cell count via cytology. Differential counts (%) were performed by a board-certified clinical pathologist who was unaware of the study purpose. Absolute BAL macrophages (cells/μL)

per mouse were calculated using BAL total white blood cell count (cells/μL) multiplied by percent macrophages.

Lung Tumor Burden Measurement

Immediately following CO_2 euthanasia, lungs were infused with 1-2 mL of India ink until fully inflated, then placed in Fekete's solution for bleaching of tumor metastases and fixation. After 24-48 hours of fixation in Fekete's solution, samples were stored in 70% ethanol until further processing. Following fixation, single sections from each lung lobe were processed and embedded into paraffin blocks using standard protocols at the University of Illinois Veterinary Diagnostic Laboratory. Five-micron tissue sections were cut in at two levels separated by 50 microns, placed onto glass slides, and stained routinely with hematoxylin and eosin (H&E). Sub-gross images of H&E stained lung tissue sections were captured at low magnification (1.25x) for measurement of tumor burden. Gross images of all lungs were collected after fixation and prior to histologic processing.

Tumor burden measurement was performed using Adobe Photoshop CC 2017.1.1 (Adobe Systems, San Jose, CA). Using an image-embedded micrometer, a measurement scale was created to designate pixel distance into a logical length (millimeters). Each lung tumor section was measured at both levels for total lung area (mm^2) using the Magnetic Lasso tool and total tumor area (mm^2) using the Magic Wand tool. Total tumor area was then divided by total lung area per section, yielding a % tumor burden per section. Percent total tumor burden per mouse was also calculated as the summation of tumor area in all sections and levels divided by the total summation of lung area in all sections and levels.

Immunohistochemistry

Five-micron tissue sections cut from lung lobes set in formalin-fixed paraffin-embedded blocks were stained for either Factor VIII (A0082) or F4/80 (cs70076) using standard procedures at the University of Illinois Veterinary Diagnostic Laboratory. Factor VIII antibody was used at a dilution of 1:1000 and F4/80 antibody at dilution of 1:700.

Survival Curves

Mice within survival curve experiments were monitored daily after tail vein injection for morbidity and mortality associated with OSA metastasis to the lungs. Mice were censored if they experienced death or were euthanized due to factors unrelated to OSA pulmonary metastasis. Mice experiencing atypically prolonged or shortened survival relative to group median survival times (MST) were removed from analysis given the low percentage (less than 5%), due to failed successful tail vein injection technique. Kaplan-Meier curves were created using GraphPad Prism version 7.0a for Mac OS X (GraphPad Software) with day 1 being designated as the day following tail vein injection. For mice experiencing euthanasia, day of death was recorded as the day following euthanasia. For mice experiencing natural death, day of death was recorded as the date found dead. Survival curves were compared in GraphPad Prism using the Log-rank (Mantel-Cox) test or the Gehan-Breslow-Wilcoxon test, as appropriate. Survival times are expressed as the median (MST).

Microscopic versus Macroscopic Lung Tumor Burden

Mice were injected with $1x10^6$ mycoplasma-negative K7M2 via tail vein (day 0), then sacrificed at daily intervals from day 2 through day 7 to evaluate when neoplastic burden in the

lung transitioned from a microscopic to macroscopic process. Lungs were collected and processed identically to those collected for lung tumor burden measurement, with the exception of a second level being cut in at 200 microns rather than 50 microns. Representative H&E images of tumor metastases were captured at 20x and 50x. Gross images of the lungs were also captured after fixation and prior to histologic processing with a dissecting microscope.

Enzyme-Linked Immunosorbent Assays

Murine-specific IFNγ, IL-2, and TNFα were measured with Quantikine ELISA (R&D Systems) using serum collected immediately post-euthanasia.

Phagocytosis Assays

Two murine immune cells lines (J774, RAW) and two canine immune cell lines (DH82, Nike) were treated with CpG ODN 2395 for 1, 12, or 24 hours, then phagocytosis was measured using the Vybrant Phagocytosis Assay Kit (Invitrogen) according to manufacturer's instructions.

Statistical Analysis

Statistical analysis was performed with GraphPad Prism version 7.0a for Mac OS X (GraphPad Software) with significance set at $p < 0.05$. All data is expressed as the mean ± SEM. Normality was assessed with the Shapiro-Wilk test. Tumor burden, cytokine, phagocytosis, and liposomal clodronate data were analyzed using one-way ANOVA with Tukey's for parametric data and the Kruskal-Wallis test with Dunn's for non-parametric data. Cytotoxicity data was assessed with repeated measures ANOVA and Tukey's post-test. For all data, $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

Results

Prophylactic TLR Treatment Reduces OS Lung Metastatic Burden and Prolongs Survival Time

To assess the activity of our 3 selected TLR agonists (Pam3CSK4, Poly(I:C), and CpG ODN 2395) in a relevant model of murine metastatic OS, we employed a prophylactic treatment scheme whereby mice were "vaccinated" weekly over a 4-week period with their assigned TLR agonist prior to K7M2 tail vein injection, in an effort to maximize the potential for our TLR agonists to optimally stimulate the immune system and increase the likelihood of observing a positive effect in regards to metastatic OS lung tumor burden and survival time (**Figure 3.1**). In addition, we also chose to initially investigate, besides evaluating the efficacy of single agent TLR vaccination alone (**Figures 3.2-3.5**), whether concurrent TLR vaccination along with a K7M2 tumor cell lysate would be beneficial in enhancing anti-tumor immunity through the induction of a more robust adaptive immune response (**Figure 3.6**).

Lung tumor burden from mice receiving single agent therapy and sacrificed at either 14- or 21-days post-K7M2 injection showed marked variability in the capacity for TLR agonists to exert *in vivo* efficacy for the prevention of OS metastatic disease (**Figures 3.2, 3.4**). Mice receiving either single agent Poly(I:C) or CpG ODN 2395 exhibited gross reductions at the 14- (**Figure 3.2a**) and 21-day (**Figure 3.4a**) time periods, while Pam3CSK4 treated mice had lung tumor burden grossly similar to the control mice at both time periods. Histologically, mice receiving CpG ODN 2395 also had markedly decreased total lung tumor burden (**Figure 3.2b**) and decreased tumor burden per lung section (**Figure 3.2c**) when compared to control mice and Pam3CSK4 mice at day 14, corroborating the gross reductions observed. Similar results were noted for Poly(I:C) mice in comparison to control and Pam3CSK4 tumor burden per lung section

(**Figure 3.2c**), however, total tumor burden was not significant when compared to controls, but was still decreased in comparison to Pam3CSK4 (**Figure 3.2b**). Although grossly, Poly(I:C) and CpG ODN 2395 therapies could not be appreciated to have discernable gross differences in tumor burden, CpG ODN 2395 therapy did result in lesser amounts of tumor burden per lung section in comparison to Poly(I:C) (**Figure 3.2c**). Circulating cytokine levels in these mice revealed an increase in IFNγ for mice receiving CpG ODN 2395 only (**Figure 3.3**). Circulating IL-2 and TNFα levels were not different between groups (data not shown).

The findings at day 14-post K7M2 injection were similar overall in comparison to day 21 sacrifice periods (**Figure 3.4b, c**), with few exceptions. Total tumor burden for Pam3CSK4 was not different comparatively to Poly(I:C) or CpG ODN 2395 at this time point (**Figure 3.4b**), whereas it was increased at day 14. Another item of interest was that relative Poly(I:C) tumor burden per lung section was larger in comparison to CpG ODN 2395 at day 21 (**Figure 3.4c**) than at day 14. This suggested that the effects of Poly(I:C) in reducing OS lung metastasis may be marginal or short-lived, whereas CpG ODN 2395 may have more substantial long term reductions in OS lung tumor burden.

Survival times for Poly(I:C) and CpG ODN 2395 were also significantly prolonged compared to controls (**Figure 3.5**), and this finding was repeatable across 2 separate experiments. Poly(I:C) had a minimal prolongation in survival time with a MST of 31 (**Figure 3.5a**) or 41 days (**Figure 3.5b**) compared to control MST of 25 and 23 days, respectively. This consistent mild increase in survival time may be directly related to the relative elevation of Poly(I:C) lung tumor burden that was noted at later points during histologic tumor burden analysis, thus further supporting that Poly(I:C)'s efficacy in prevention of OS lung tumor metastatic burden is marginal. CpG ODN 2395, on the other hand, exhibited a clinically relevant increase in survival time of

approximately 3.7x in comparison to controls, regardless of survival experiment, with a MST of 111 days (**Figure 3.5a**) and 99 days **(Figure 3.5b**), compared to MST of 25 and 33 days for controls, respectively. CpG ODN 2395 survival times were also significantly increased compared to Poly(I:C) survival (p = 0.01 for survival curves). Pam3CSK4 did not extend survival time relative to controls in either experiment.

While single agent therapy with CpG ODN 2395 clearly reduces OS metastatic tumor burden within the lungs and increases survival time to a clinically relevant level, we wondered if concurrent vaccination with a sterile K7M2 tumor cell lysate would influence survival times through priming of the immune system with tumor-associated antigens and hence augment a robust adaptive immune response (**Figure 3.6**). While gross reductions in lung tumor burden (**Figure 3.6a**) were similar in conjunction with K7M2 tumor cell lysate for Poly(I:C) and CpG ODN 2395, an apparent increase in the Pam3CSK4 + lysate group was noted in comparison to the lysate only group. This was also characterized by significant increases in tumor burden per lung section for Pam3CSK4 + lysate in comparison to both the control and lysate only groups (**Figure 3.6c**), but was not appreciable on a total tumor burden level (**Figure 3.6b**) and did not change survival time relative to the control group (**Figure 3.6c**). Survival time trends were also similar to single agent therapy results amongst the individual agonists (**Figure 3.6c**), although the MST for Poly(I:C) + lysate (53 days) was approximately 23 days longer than the average of single agent therapy (average of 36 days). CpG ODN 2395 + lysate MST was conversely blunted by 32 days (73 day MST v. average 105 days for single agent therapy). K7M2 tumor cell lysate alone also appeared to have a mild effect on survival time (MST of 34 days v. 25 days for control). While it was considered too laborious and impracticable from a clinical aspect to pursue concurrent lysate treatment in future experiments, concurrent tumor lysate vaccination with TLR agonists do not

clearly improve upon anti-cancer activities, which suggests an augmented adaptive immune response may not be the underlying reason for enhanced survival time in some of the treatment groups.

CpG ODN Promotes a CTL and T_h1 Response in Tumor-Bearing Mice

In addition to evaluating tumor burden and survival secondary to TLR agonist treatment, we also sought to characterize the anti-tumor immune response in TLR-treated, OS-bearing mice through evaluation of splenocyte lymphocyte subpopulations via flow cytometry at day 14 and 28 post-K7M2 injection (**Figures 3.7-3.8**) as well as *ex vivo* cytotoxicity assays from splenocytes harvested 14 days after tumor injection (**Figure 3.9**). Splenic weight was consistently increased in CpG ODN 2395 mice compared to controls at both day 14 (**Figure 3.7a)** and day 28 (**Figure 3.8a**); splenic weight was also higher compared to Pam3CSK4 and Poly(I:C) at day 14. Poly(I:C) had an elevated splenic weight compared to controls at day 14, while Pam3CSK4 splenic was higher at day 28. These elevations in splenic weight were often contributed to increased mononuclear cell populations, based off Histopaque pellet size and cell counts. Histologically, all spleens were consistent with extramedullary hematopoiesis. Total spleen count for $CD3^+/CD8^+$ cytotoxic T lymphocytes (CTLs) (**Figure 3.7b**) and $CD3^+/CD8^+/CD25^+$ activated CTLs (**Figure 3.7c**) were significantly increased in the CpG ODN 2395 group at day 14; this effect was lost at day 28 (**Figures 3.8b, c**). $CD3^+/CD4^+/CXCR3^+$ T_h1-polarized T cells were significantly increased for CpG ODN 2395 when compared to PamCSK4, but were not elevated compared to controls (p = 0.07) **(Figure 3.7c**). This effect was also lost at day 28 (**Figure 3.8d**). No changes in T regulatory lymphocytes (**Figures 3.7e and 3.8e**) or NK cells (**Figures 3.7f and 3.8f**) were noted for any group at either time point. From this, CpG ODN 2395 was determined to influence an

effector cell response characterized by increased CTLs and T_h1 cells, both of which are highly recognized as being influential in anti-tumor immunity.

We also wondered whether TLR agonist treatment could enhance specific cell-mediated cytotoxicity against the K7M2 OS tumor cell line. To investigate this objective, we cultured splenocytes from TLR-treated, OS-bearing mice 14 days after K7M2 tail vein injection and subsequently cultured the mononuclear cell population with mitomycin-c treated K7M2 cells for 7 days in the presence of rmIL-2. These cells were then employed within an *ex vivo* cytotoxicity assay to determine if the effector cells displayed specific cytotoxicity against K7M2. The Balb/3T3 fibroblast line was used as the non-specific control target. Poly(I:C) treatment significantly increased cytotoxicity at effector:target (E:T) ratios of 25:1, 50:1, and 100:1 compared to control mice. However, the cytotoxicity levels were similar between K7M2 and Balb/3T3, indicating non-specific cytotoxicity (**Figure 3.9b**). Pam3CSK4 also exhibited increased non-specific cytotoxicity at the 100:1 E:T ratio (**Figure 3.9a**). CpG ODN did not statistically increase cytotoxicity at any ratio, but at the 100:1 E:T ratio did start to show some divergence from non-treated mice, suggesting CpG ODN 2395 may enhance non-specific cytotoxicity at high concentrations (**Figure 3.9c**). This suggests that while TLR agonists may enhance non-specific cytotoxicity in effector cells, it is unlikely to play a significant role in the reduction in metastatic OS lung tumor burden observed with CpG ODN 2395. We did not further investigate if this cytotoxicity was important for the reduction in tumor burden with Poly(I:C).

CpG ODN Therapy May Be Effective in a Setting of Micrometastatic Disease

While CpG ODN 2395 appeared to be a promising immunotherapeutic based off our findings within the prophylactic treatment scheme, this was an impractical model to assess how

CpG ODN 2395 might function in a spontaneous OS setting, where prediction of OS development is improbable. Therefore, we evaluated the administration of CpG ODN 2395 in an "accelerated" treatment scheme, where CpG ODN 2395 was given either around the time of K7M2 OS tail vein injection or several days after tail vein injection, to better replicate a true canine OS clinical scenario, whereby pet dogs present for adjuvant therapeutic management in the micrometastatic pulmonary setting.

Surprisingly, CpG ODN 2395 given the same day (day 0) as K7M2 tail vein injection decreased lung tumor burden and resulted in an approximately 3x increase in survival time when compared to control mice, as well as when compared to mice receiving CpG ODN therapy one week after tumor injection (**Figure 3.10-3.11**). This was apparent not only grossly (**Figure 3.10a**) but also histologically (**Figure 3.11b, c**). However, initiating CpG ODN 2395 treatment 7 days after tumor cell injection was ineffective at increasing survival time (**Figure 3.11**), even though histologic tumor burden in the day 7 group was decreased compared to controls (**Figure 3.10b, c**). Similarly, intervening with CpG ODN 2395 earlier than day 7 (e.g., day 3 or 5 post-tumor cell injection) also did not improve survival time from a clinically-relevant standpoint (**Figure 3.11b**).

Although K7M2 cells injected intravenously reach the lung parenchyma within 1 hour,[4] we were curious if the lack of CpG ODN's efficacy at later time points correlated with a transition from microscopic to macroscopic tumor burden. To investigate this, we injected naïve mice with K7M2 and sacrificed them on a daily basis starting 48 hours after tumor injection. Macroscopic tumor metastases could be observed grossly with magnification starting as early as day 3 after tumor injection and became visible to the naked eye by day 5 **(Figure 3.12**). Microscopically, tumor emboli were easily observed at day 2 and tumor cells also appeared to have extravasated at this time (**Figure 3.13**). This suspicion was confirmed with Factor VIII immunohistochemistry

(images not shown). As time progressed, metastases became larger and more organized, with clearly defined extravasation on H&E by days 3-4. While we did not investigate CpG ODN 2395 therapy 1-2 days after tumor cell injection, the timeline of OS tumor progression within the lungs suggests CpG ODN 2395's usefulness may be limited to the very early stages of micrometastatic disease, such as initial showering of the lungs with tumor emboli. This also raises the question of whether CpG ODN 2395 exerts its anti-tumor activity in the form of a cell-mediated immune response or through non-cellular microenvironment alterations that are not conducive for lung metastasis.

Efficacy of CpG ODN is Not Dependent Solely by Macrophages

We also sought to elucidate the mechanism of action (MOA) of CpG ODN 2395. Given the apparently narrow window of efficacy for CpG ODN 2395 therapy based off the findings in our accelerated treatment scheme, we wondered if macrophage activation and cytotoxic activity may be a key MOA, especially given the robust TNFα elucidated by CpG ODN 2395 treatment (**Chapter II**). Indeed, CpG ODN 2395 treatment enhanced phagocytic activity of the murine immune cells J774 and RAW (**Figure 3.14a, b**), but did not cause consistent increases in phagocytic activity within the canine immune cells DH82 and Nike (**Figure 3.14c, d)**. Macrophage depletion with liposomal clodronate also failed to negate the efficacy of CpG ODN 2395 therapy, regardless of intranasal (IN) or intraperitoneal (IP) administration (**Figure 3.15**). Intraperitoneal clodronate therapy markedly decreased splenic weight (**Figure 3.16b**) and splenic macrophage count as analyzed by F4/80 IHC (**Figure 3.16c**), but coincidentally caused a rebound effect in lung macrophages numbers as analyzed by BAL. Intranasal clodronate also failed to

statistically decrease lung macrophage numbers (**Figure 3.16a**). This may represent suboptimal macrophage depletion levels if the site of CpG ODN's activity is localized to the lungs.

Discussion

These results conclude that the TLR9 agonist CpG ODN 2395 can successfully reduce metastatic OS tumor burden in a clinically-relevant murine model of metastatic OS and also is most effective in stimulating an immune response in OS-bearing mice, characterized by increased circulating IFNγ and increased numbers of T_h1 and cytotoxic T cells, making it a reasonable candidate TLR agonist for application in dogs with spontaneous OS. We hypothesized that TLR agonists would have variable results in this model, which was supported by the highly divergent response between Pam3CSK4, Poly(I:C), and CpG ODN 2395, where Pam3CSK4 appears pro-tumorigenic, Poly(I:C) appears to have anti-tumorigenic properties initially that are later lost, and CpG ODN 2395 has the best overall effect with the largest increase in survival time. However, the results from this chapter also prove the effectiveness of our systematic investigation, which did initially suggest CpG ODN 2395 as having the most minimal pro-tumorigenic activity in **Chapter II**.

Interestingly, the activity of CpG ODN 2395 is limited to a short time frame between tumor cell injection and micrometastasis development, based off observing only mild increases in survival time when CpG ODN 2395 was administered starting day 3 after tumor cell injection and interpreted in combination with the histologic findings of neoplastic OS cell extravasation into the lung parenchyma as early as 48 hours after tumor cell injection in naïve mice. Ideally, it would be interesting to look at CpG ODN 2395's efficacy at days 1 and 2 post-tumor injection. If CpG ODN 2395 therapy at these time points is also ineffective, then it is possible CpG ODN 2395 is

exerting its anti-tumor effects from a cytokine level,[5] which may be directly causing cancer cell death during metastasis or altering tumor cell expression of metastasis-related genes necessary for extravasation into the lung parenchyma. Based off our data in **Chapter II**, it is possible CpG ODN 2395's effect could be through TNFα release. CpG ODN 2395's activity could also be mediated by NK cells,[6] given the increase in circulating IFNγ in CpG-treated mice, even though we did not appreciate increased NK cell splenic numbers. Alternatively, CpG ODN 2395 may be acting directly on OS cells, either through activation of anoikis to prevent metastasis or through inhibition of signaling pathways, such as decreased pAkt,[7,8] which was observed in K7M2 after treatment with CpG ODN 2395 in **Chapter II**. Phosphorothioate CpG's have been shown to inhibit cellular adhesion in melanoma[9] and if this mechanism is functional in OS, then it may be related to altered ezrin or ß4 integrin signaling, both of which are important in OS anoikis resistance.[10-12] These theories could explain the swift time frame that CpG ODN 2395 appears to function within, since it on average takes 4-7 days for an adaptive immune response to develop.[13]

While we were unable to show specific cell-mediated cytotoxicity was triggered by CpG ODN 2395 and that macrophages are unlikely to be the sole critical component to CpG ODN 2395's efficacy, we still cannot definitely conclude that the anti-tumor response observed is not directly cell-mediated, even though the elicited response is apparently swift in our accelerated model of CpG ODN 2395 therapy. We specifically chose to explore whether macrophage depletion would reverse the effects of CpG ODN 2395, as macrophages can not only be directly cytotoxic to tumor cells,[14] but also promote adaptive anti-tumor immunity and active innate NK cells.[15,16] While we observed no reversal of lung tumor burden with macrophage depletion using liposomal clodronate, it is questionable as to whether our macrophage depletion was adequate, given the lack of significant depletion of splenic macrophages in the IN group and the rebound

effect observed in BAL macrophages in the IP group. To better explore the cell-mediated role of CpG ODN 2395 in prevention of OS lung metastasis, additional immune cell depletion studies would need to be performed and may be essential for a better understanding of CpG ODN 2395's potential as an immunotherapeutic for OS.

FIGURES AND TABLE

Lymphocyte Subpopulation	Antibody-Fluorochrome Conjugates
CTL	CD3ε-FITC CD8α-PE CD25-APC
T_h1	CD3ε-FITC CD4-PE CXCR3-APC
T_{reg}	CD4-FITC CD25-PE FoxP3-APC
NK	CD3ε-FITC CD49b-PE NKG2D-APC

Table 3.1: Antibody-fluorochrome conjugates used for the assessment of specific splenic lymphocyte subpopulations.
Three-color flow cytometry for splenic lymphocyte subpopulations included evaluation of cytotoxic T cells (CTL), T_h1-polarized T cells (T_h1), T regulatory cells (T_{reg}), and natural killer cells (NK).

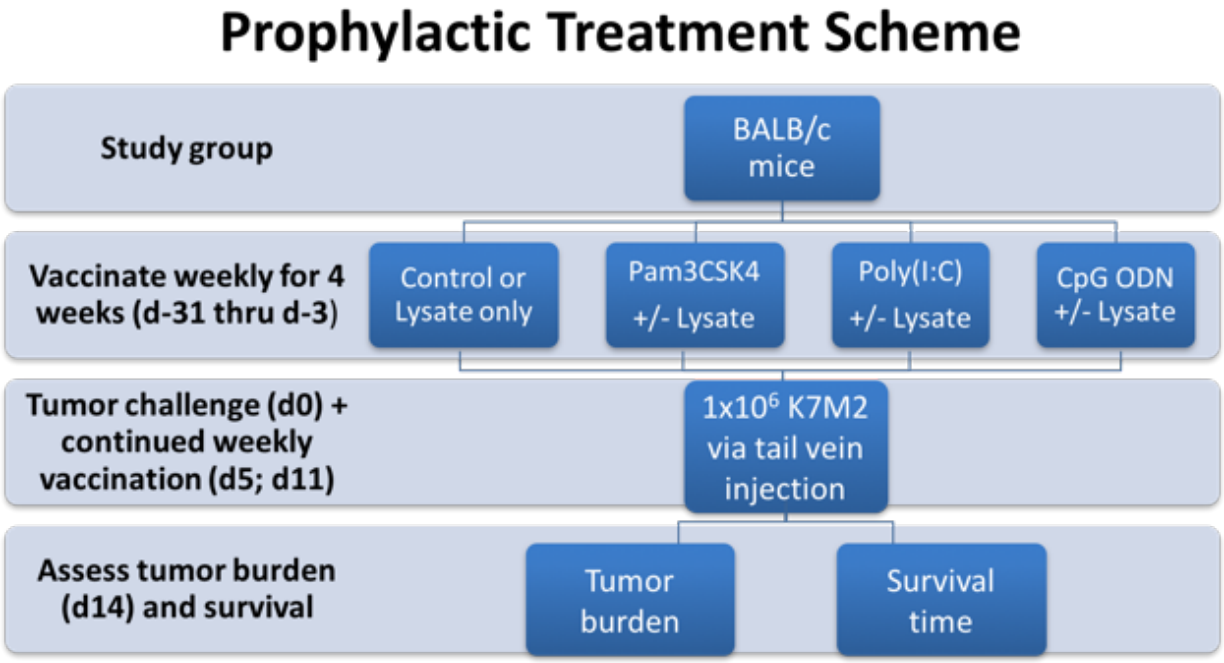

Figure 3.1: Prophylactic TLR treatment scheme utilized within a clinically-relevant murine model of metastatic OS.
Mice were injected weekly with a single TLR agonist ± K7M2 tumor cell lysate for 4 weeks prior to K7M2 tail vein injection. Two additional weekly treatments were then administered prior to sacrifice at day 14 post-K7M2 injection. Mice that were sacrificed at later time-points (e.g., day 21 or day 28) received additional weekly treatments up until the time of sacrifice.

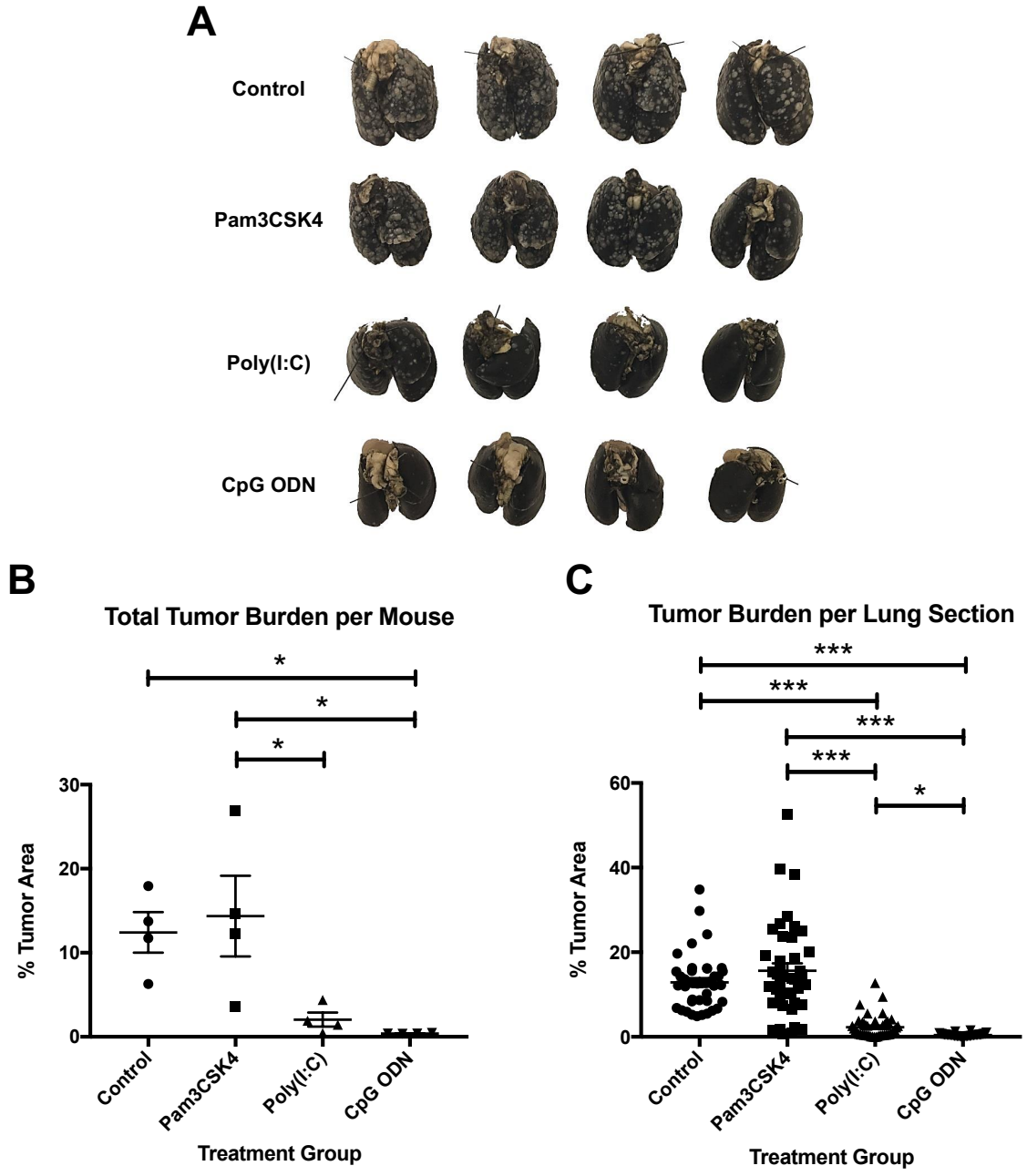

Figure 3.2: Prophylactic TLR tumor burden at day 14 post-K7M2 injection.
(A) Mouse lungs infused with India ink and fixed with Fekete's solution. Tumor metastases are visualized in white. **(B)** Total tumor and **(C)** tumor burden per lung section as measured histologically. Poly(I:C) and CpG ODN 2395 both visibly and histologically decrease metastatic OS lung tumor burden, while Pam3CSK4 tumor burden is comparatively increased. One-way ANOVA. *p<0.05 and ***p<0.001

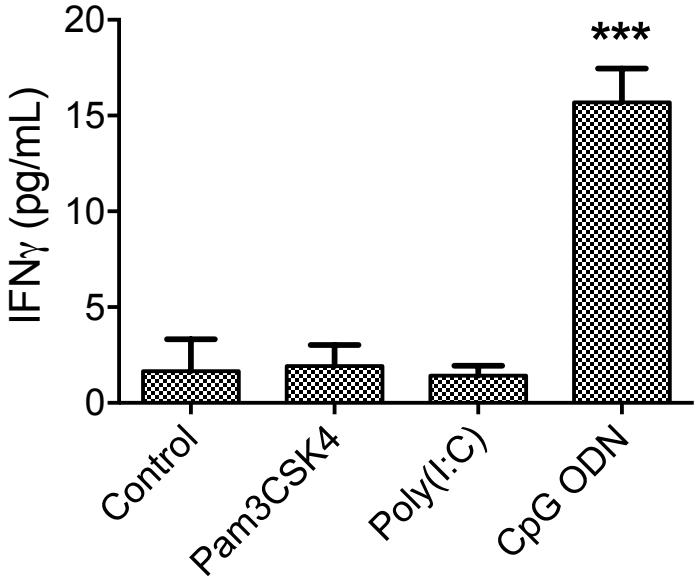

Figure 3.3: Circulating IFNγ levels in mice within the prophylactic TLR treatment scheme at day 14 post-K7M2 injection.
IFNγ is only elevated in mice receiving CpG ODN therapy. No differences in circulating IL-2 or TNFα were observed. ***$p<0.001$

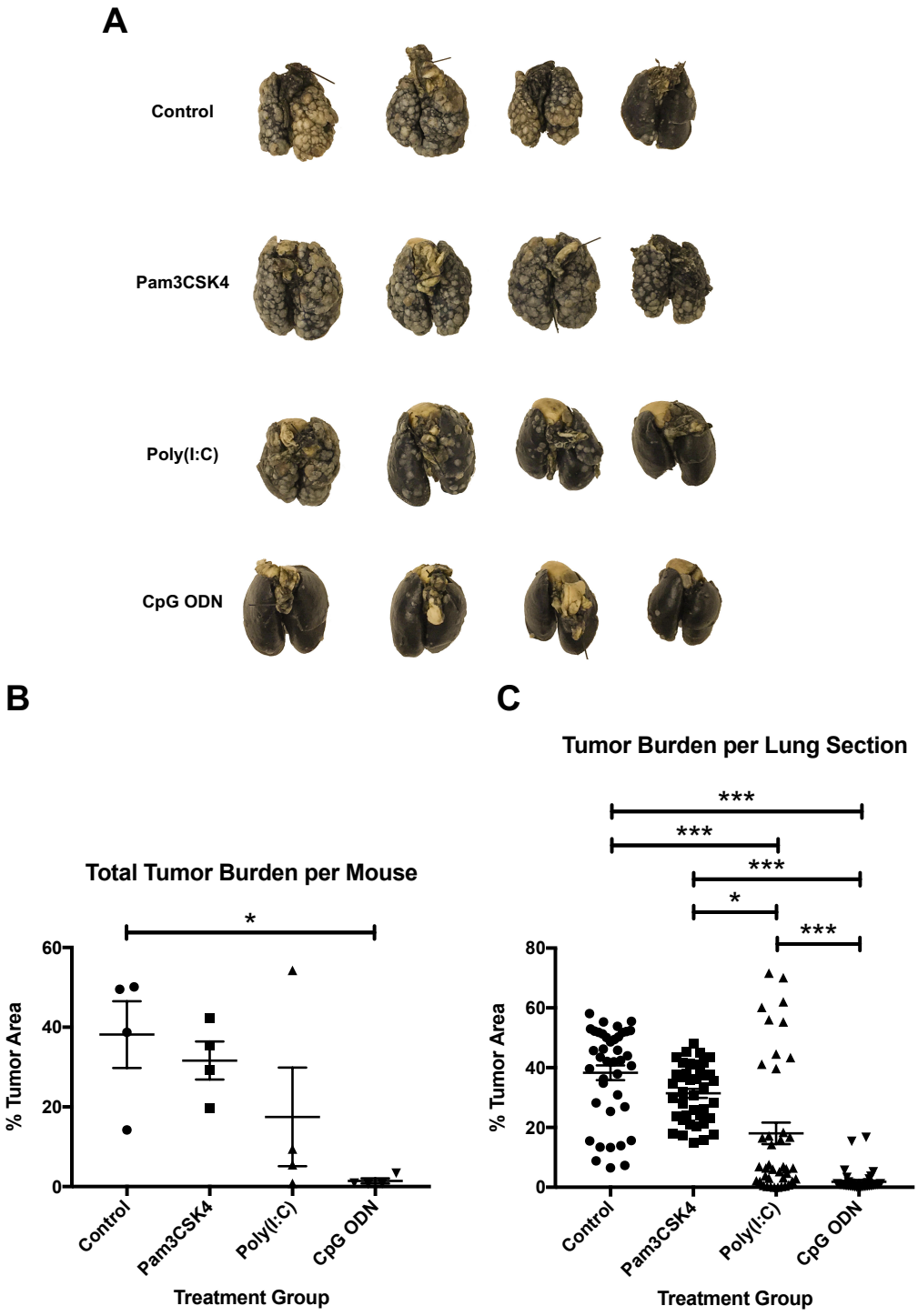

Figure 3.4: Prophylactic TLR tumor burden at day 21 post-K7M2 injection.
(A) Mouse lungs infused with India ink and fixed with Fekete's solution. Tumor metastases are visualized in white. **(B)** Total tumor and **(C)** tumor burden per lung section as measured histologically. Similar results are observed in comparison to day 14 sacrifice periods; however, Poly(I:C) tumor burden is now larger than CpG ODN 2395 **(C)**. One-way ANOVA. *p<0.05 and ***p<0.001

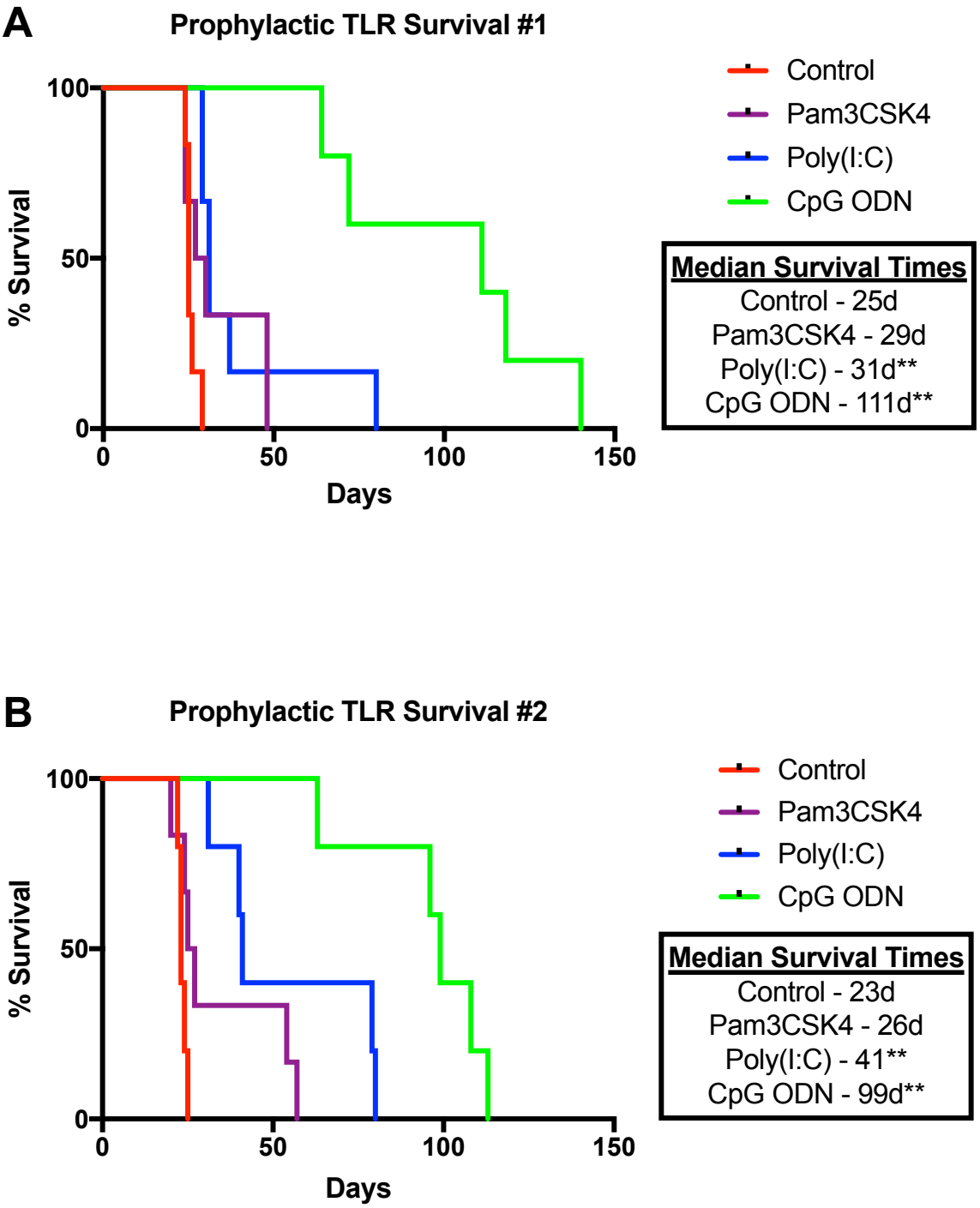

Figure 3.5: Survival curves for prophylactic TLR treatment.
(A) Initial and **(B)** repeat survival curves. Poly(I:C) and CpG ODN 2395 consistently increase survival compared to controls, but the MST (111 and 99 days) reached by CpG ODN 2395 is clinically relevant, whereas the MST (31 and 41 days) reached by Poly(I:C) is not. CpG ODN 2395 survival is also significantly longer than Poly(I:C) for both experiments (p=0.01). **p<0.01

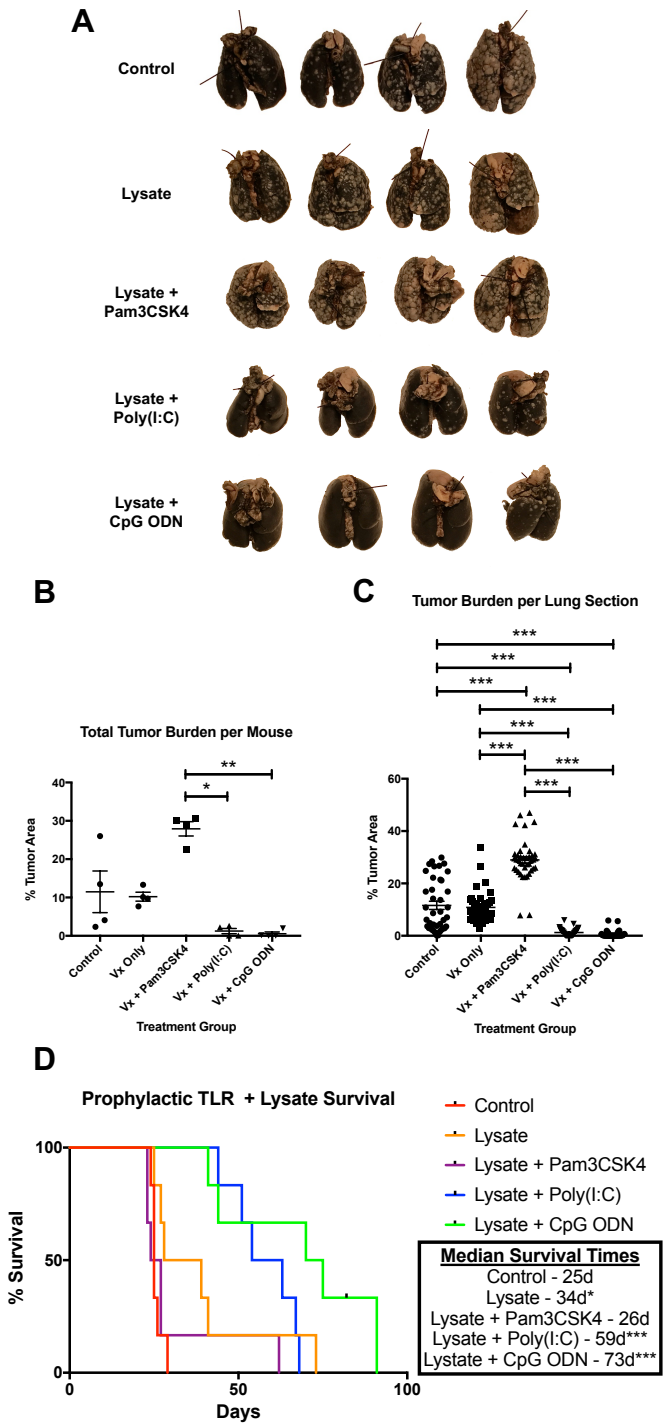

Figure 3.6: Survival curves for prophylactic TLR + K7M2 tumor lysate treatment. **(A)** Mouse lungs infused with India ink and fixed with Fekete's solution. Tumor metastases are visualized in white. **(B)** Total tumor and **(C)** tumor burden per lung section as measured histologically. **(D)** Survival of TLR + tumor lysate groups. Tumor burden trends and survival are similar to single agent TLR therapy, but lysate therapy appears to have divergent effects depending on type of administration. One-way ANOVA. *p<0.05, **p<0.01, and ***p<0.001

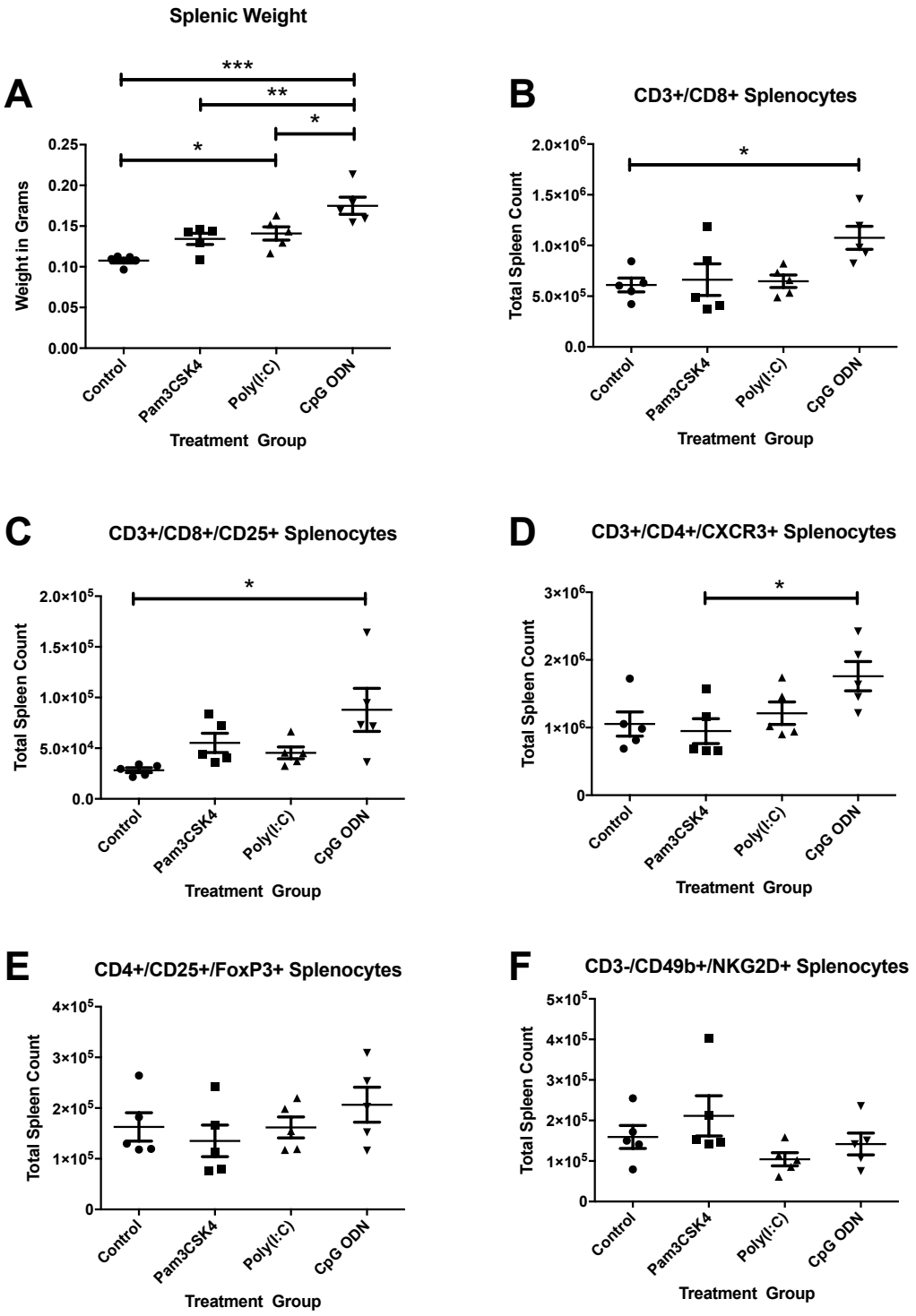

Figure 3.7: Splenic lymphocyte subpopulations at 14 days post-K7M2 injection. **(A)** Splenic weight is significantly higher for Poly(I:C) and CpG ODN 2395 compared to controls. CTLs **(B)**, activated CTLs **(C)**, and T_h1-polarized T cells **(D)** are increased only with CpG ODN 2395 therapy. T_{regs} **(E)** and NK cells **(F)** are not different between groups. One-way ANOVA. *p<0.05, **p<0.01, and ***p<0.001

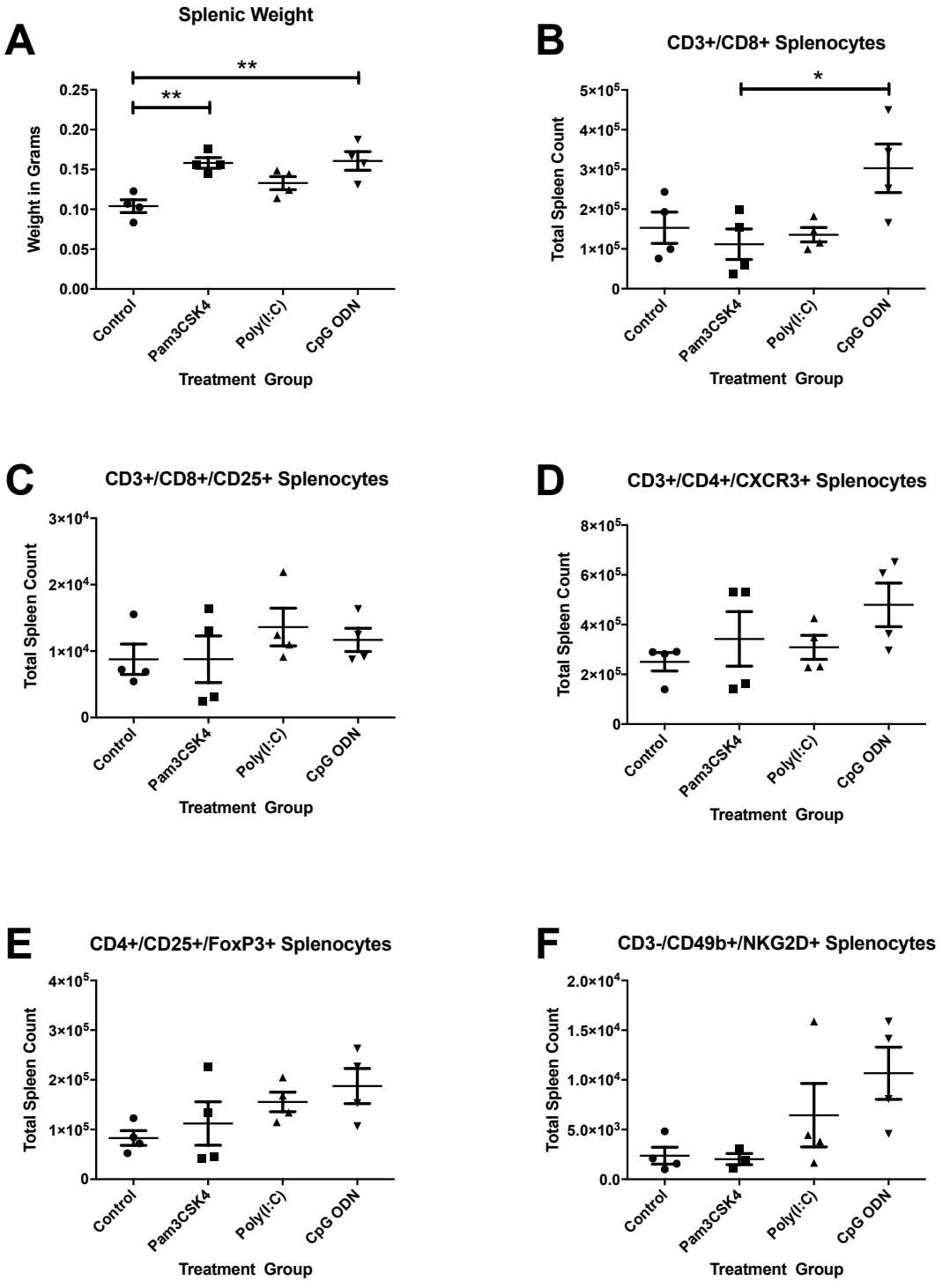

Figure 3.8: Splenic lymphocyte subpopulations at 28 days post-K7M2 injection. **(A)** Splenic weight is significantly higher for Pam3CSK4 and CpG ODN 2395 compared to controls. Most effects seen at day 14 are lost, with CTLs **(B)** for CpG ODN 2395 only being increased compared to Pam3CSK4. Activated CTLs **(C)**, T_h1-polarized T cells **(D)**, T_{regs} **(E),** and NK cells **(F)** do not different between groups. One-way ANOVA. *p<0.05, **p<0.01, and ***p<0.001

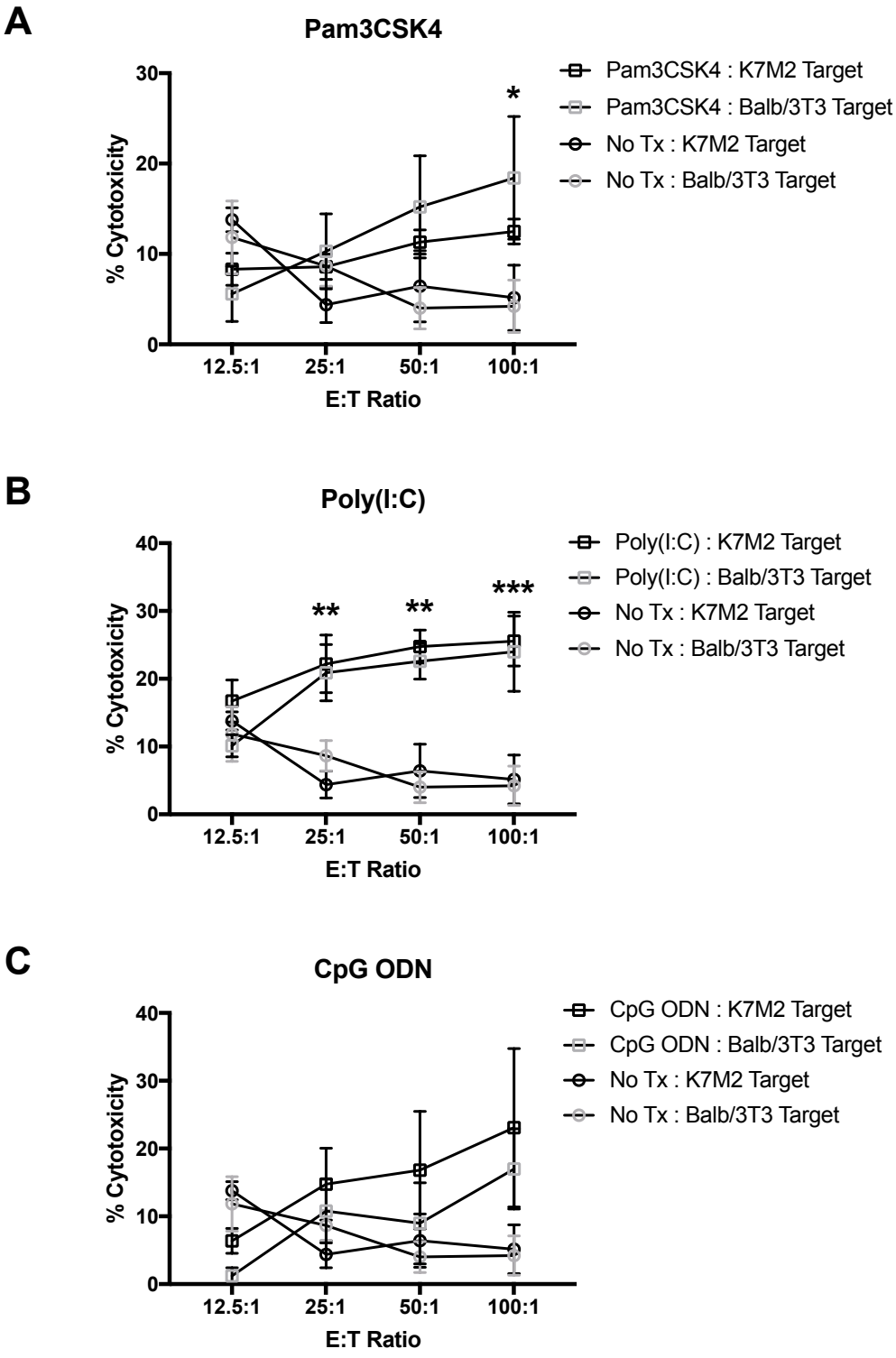

Figure 3.9: *Ex vivo* cytotoxicity assays from splenocytes collected at day 14 post-K7M2 injection under the prophylactic TLR treatment scheme.
(A) Pam3CSK4 and **(B)** Poly(I:C) both show increased cytotoxicity compared to untreated controls, but this cytotoxicity is non-specific in nature. **(C)** CpG ODN 2395 does not significantly increase cytotoxicity, although an increased trend is observed. Repeated measures ANOVA. *p<0.05, **p<0.01, and ***p<0.001

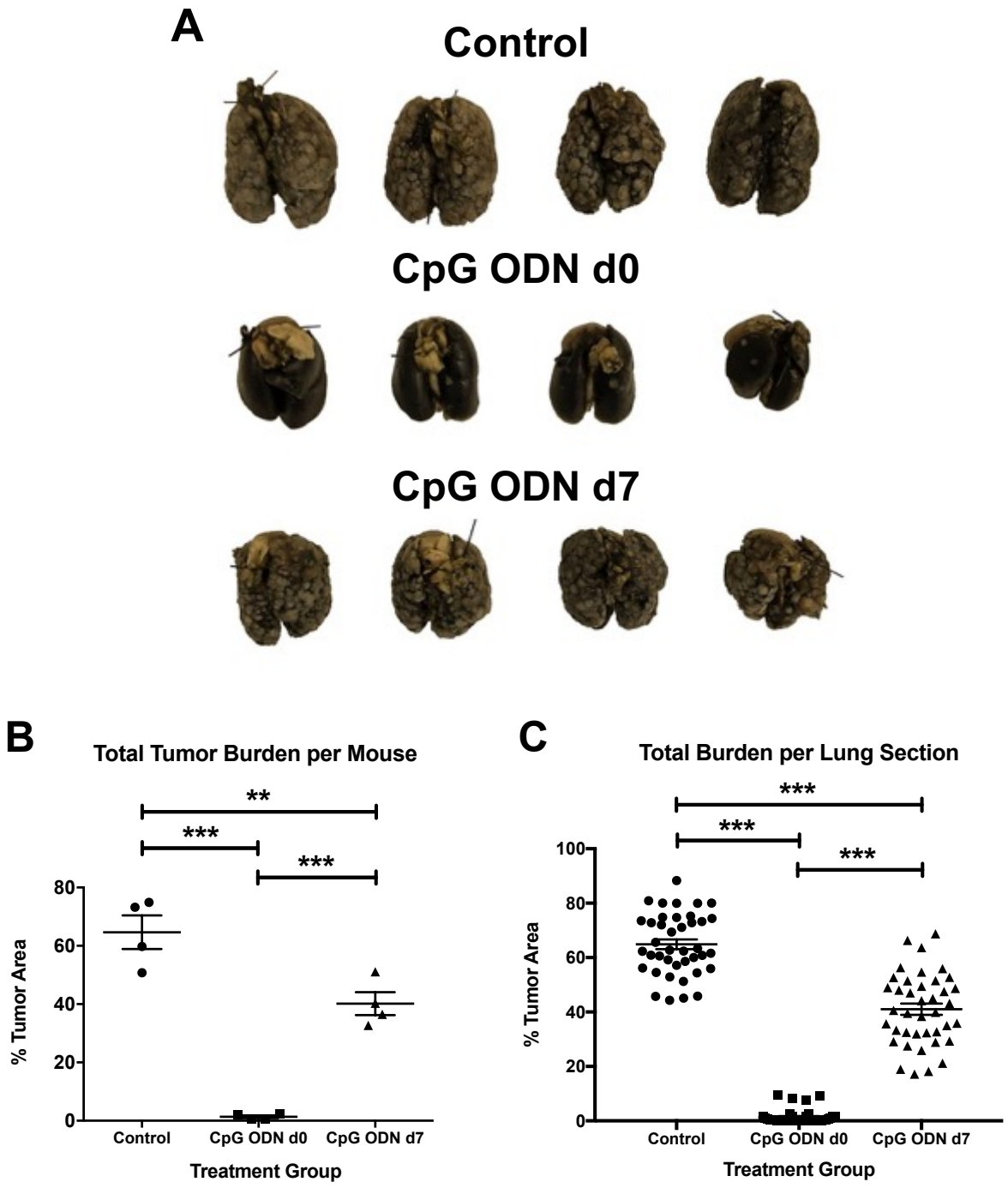

Figure 3.10: Accelerated CpG ODN 2395 tumor burden.
CpG ODN 2395 retains efficacy in the reduction of OS lung tumor burden when given the same day as K7M2 injection, but the effect is partially lost when treatment starts one week after tumor injection. **(A)** Gross tumor burden visualized via India ink and Fekete's fixation. **(B)** Total tumor and **(C)** total tumor burden per lung section. One-way ANOVA. $^{**}p<0.01$ and $^{***}p<0.001$

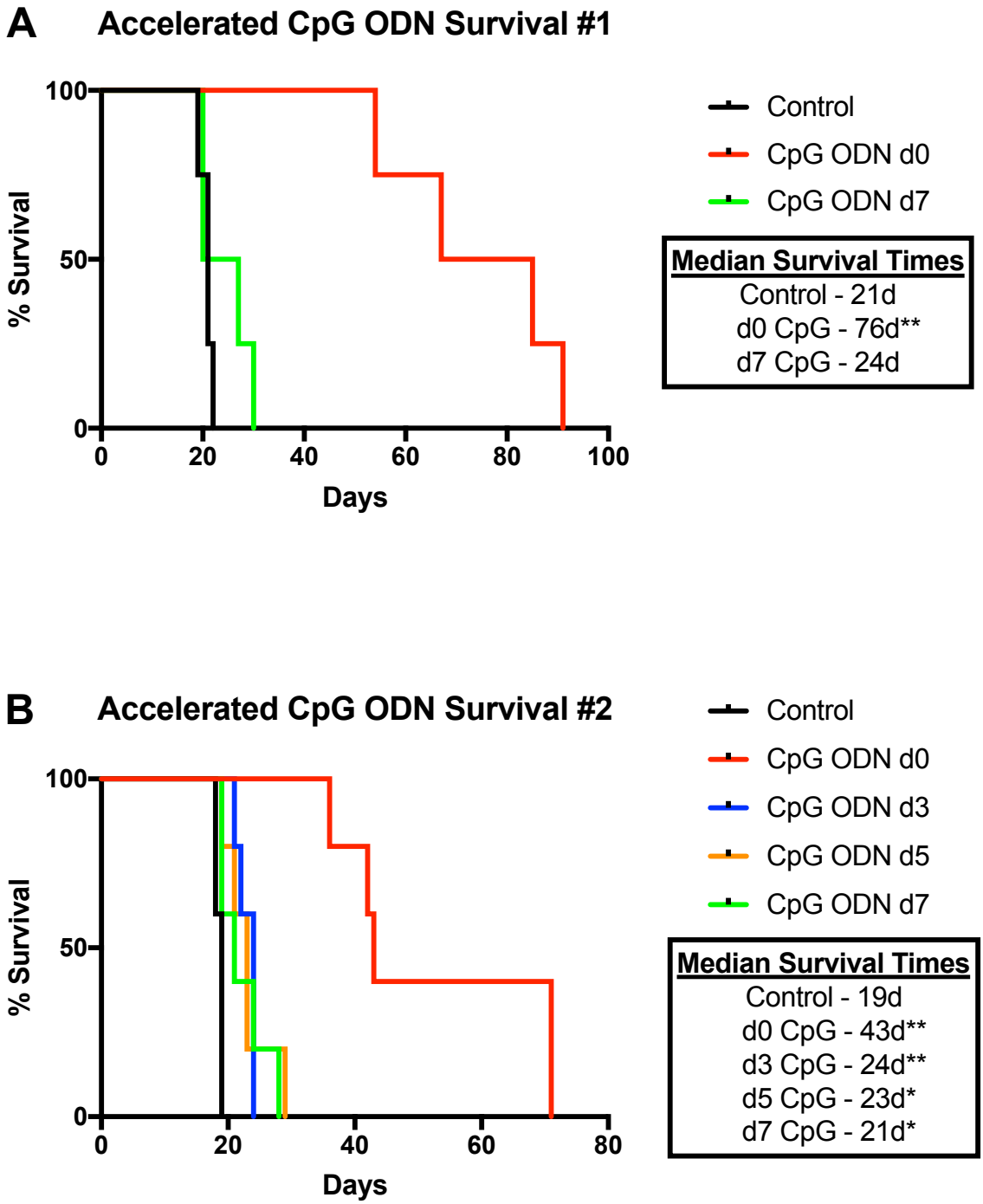

Figure 3.11: Accelerated CpG ODN 2395 survival.
(A) Initial and **(B)** repeat survival curves. CpG ODN significantly increases survival compared to controls when administered on the same day as K7M2-injection, but does not impart clinically-relevant increases in survival when intervention occurs at day 3, 5, or 7 post-tumor injection. *p<0.05 and **p<0.01

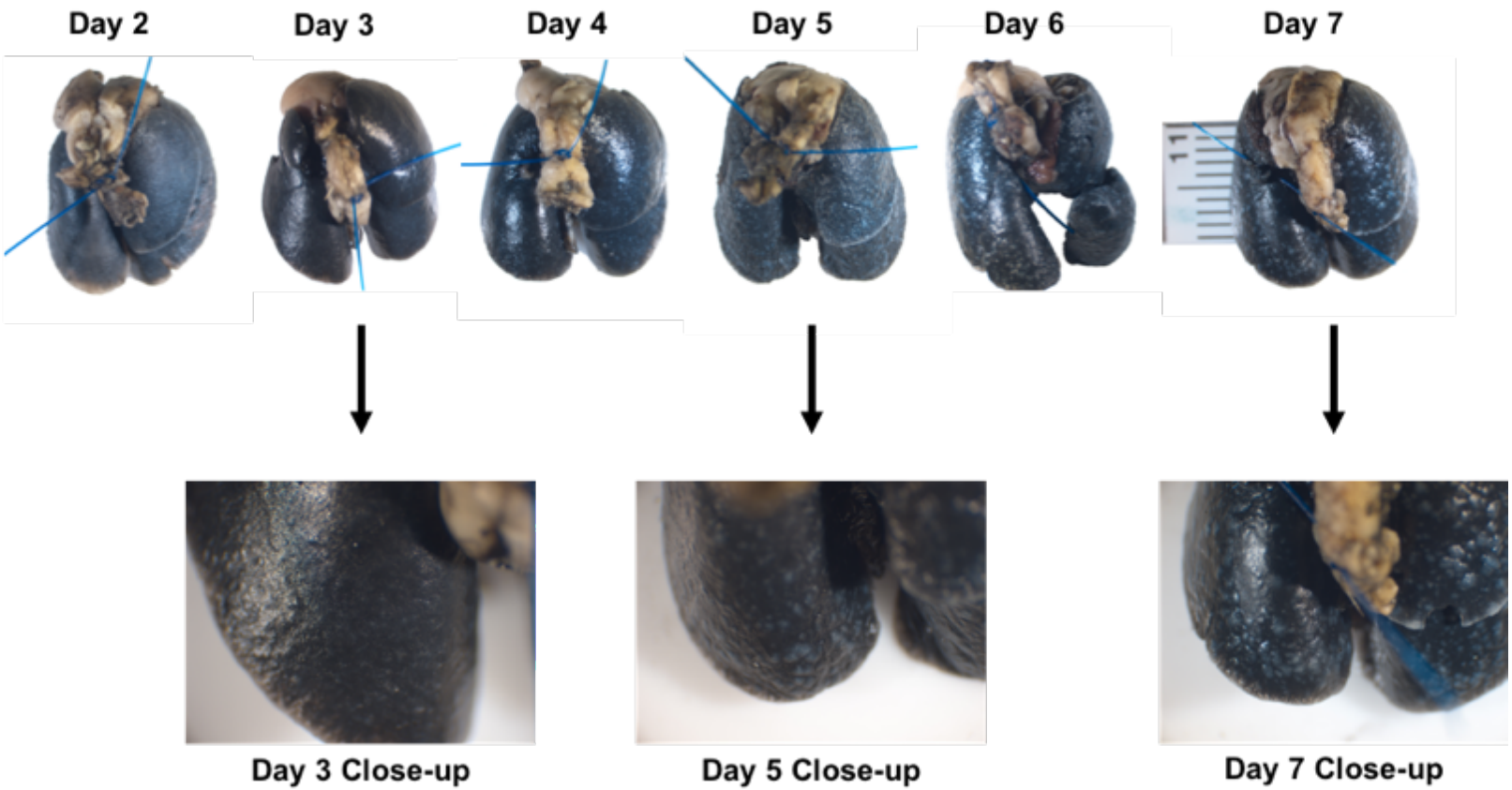

Figure 3.12: Macroscopic tumor burden.
Naïve mice injected with K7M2 were sacrificed beginning 48 hours after injection and then daily for up to 7 days post-injection. Gross parenchymal tumor metastases become consistently visible with magnification by day 3, but are not easily visible to the naked eye until day 5. Close-up images were captured using a dissecting microscope.

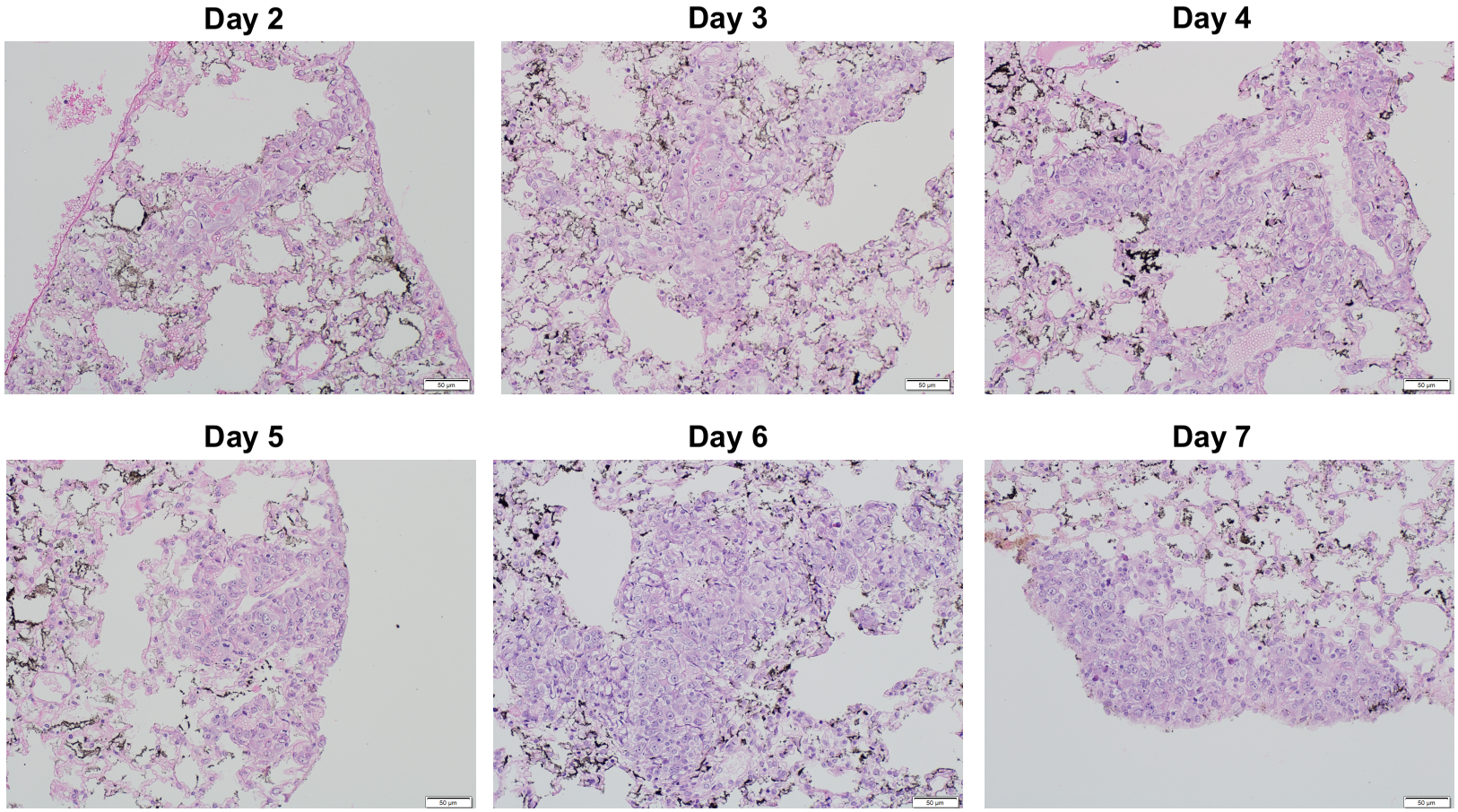

Figure 3.13: Microscopic tumor burden.
H&E sections from the lungs shown in Figure 3.12. Microscopic metastases are appreciable by day 2 and are often found perivascularly, but neoplastic cells have already extravasated at this time point. This observation was confirmed with Factor VIII IHC. As time progresses, metastases become larger and more organized.

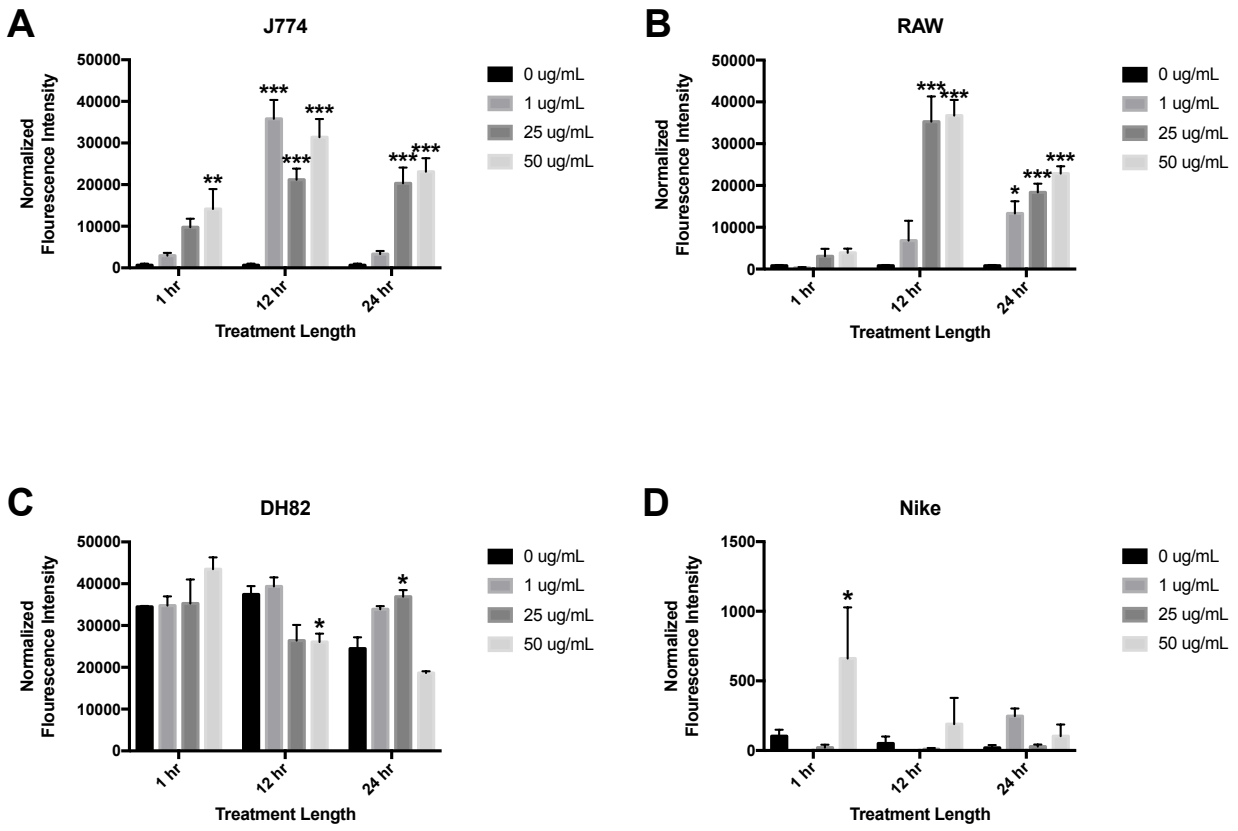

Figure 3.14: CpG ODN 2395 enhances phagocytosis in murine immune cells.
Murine immune cells **(A)** J774 and **(B)** RAW exhibit increased levels of phagocytosis with varying concentration and incubation times in response to CpG ODN 2395 treatment. Canine immune cells **(C)** DH82 and **(D)** Nike rarely show increased phagocytosis. *p<0.05, **p<0.01, and ***p<0.001

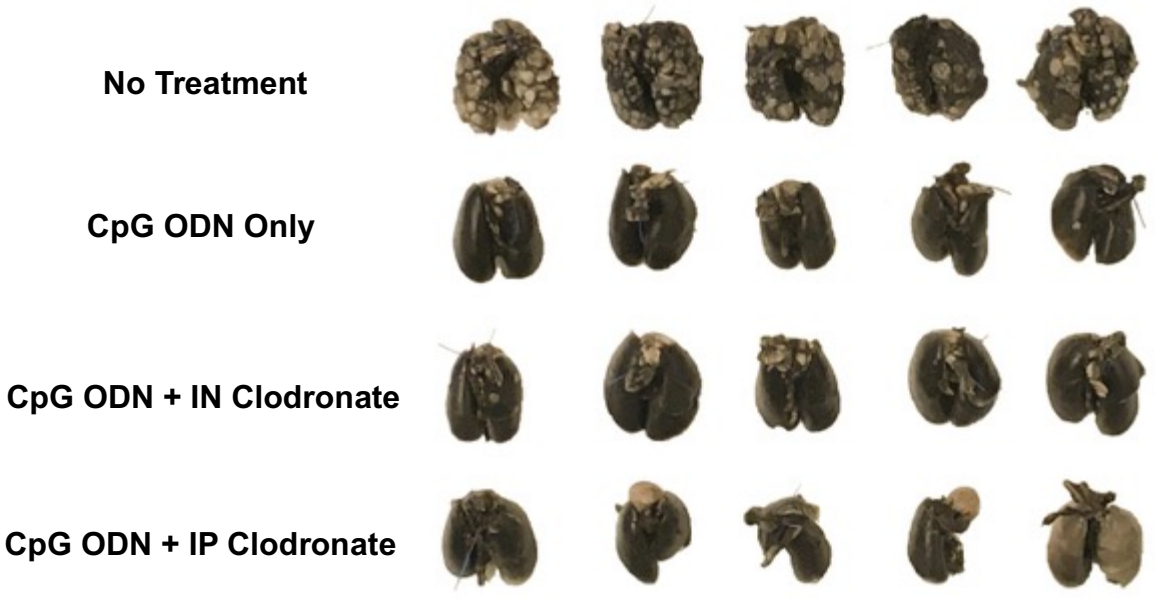

Figure 3.15: Clodronate depletion of macrophages does not reverse the effect of CpG ODN 2395 in reducing lung metastases.
Macrophage depletion with liposomal clodronate does not reverse the reduction of lung tumor burden observed with CpG ODN 2395 therapy, regardless of the route of clodronate administration (intranasal versus intraperitoneal).

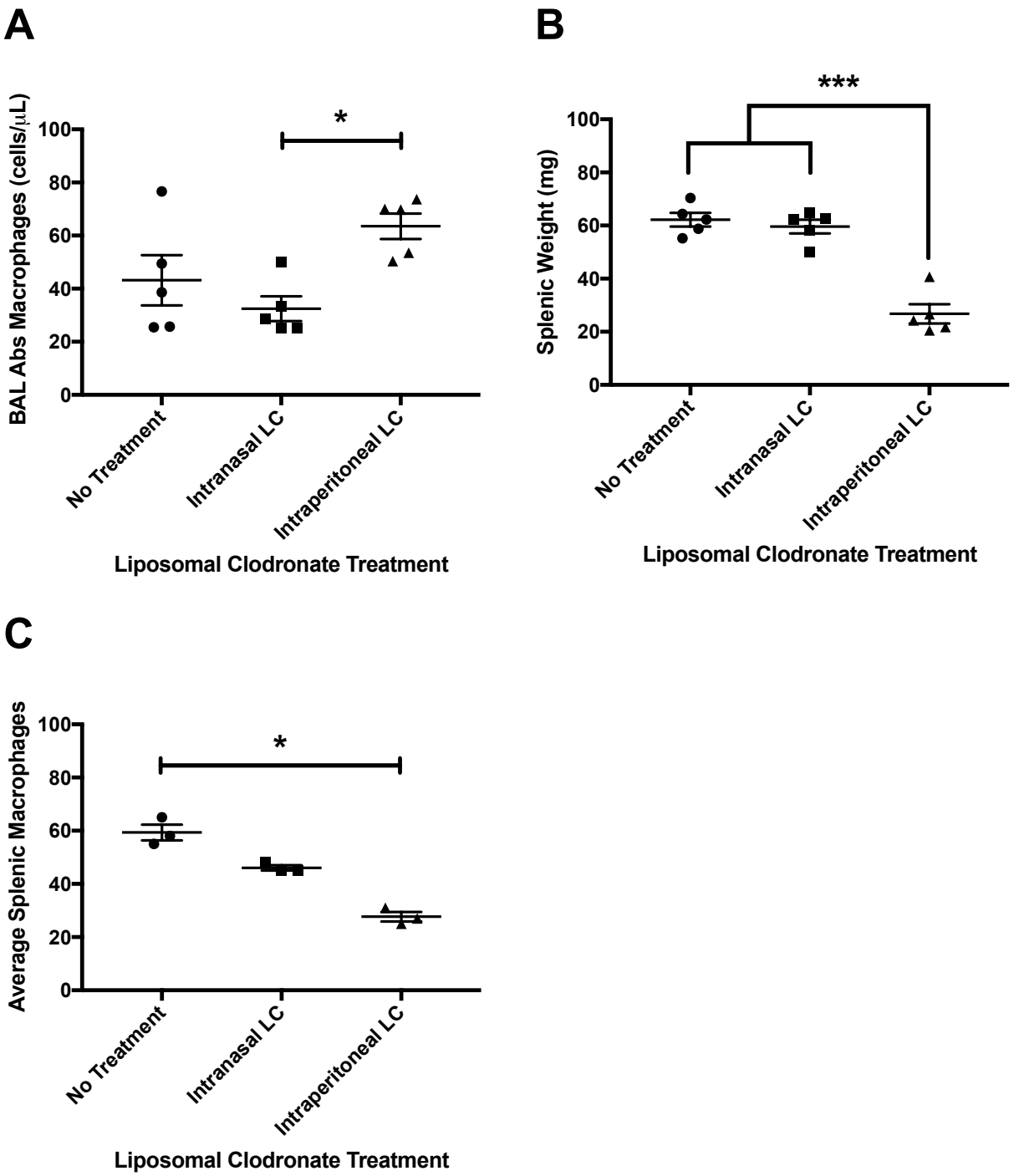

Figure 3.16: Effectiveness of clodronate on macrophage depletion.
Clodronate does not statistically decrease lung macrophages compared to controls, although decreases were observed with intranasal administration **(A)**. Splenic weight **(B)** and splenic macrophages **(C)** were markedly decreased in mice treated with intraperitoneal clodronate, which was characterized by marked red pulp depletion. One-way ANOVA. *p<0.05 and ***p<0.001

LITERATURE CITED

1. Khanna C, Prehn J, Yeung C, et al. An orthotopic model of murine osteosarcoma with clonally related variants differing in pulmonary metastatic potential. Clin Exp Metastasis 2000;18:261-271.

2. Ek ETH, Dass CR, Choong PFM. Commonly used mouse models of osteosarcoma. Crit Rev Oncol Hematol 2006;60:1-8.

3. Sottnik JL, Campbell B, Mehra R, et al. Osteocytes serve as a progenitor cell of osteosarcoma. J Cell Biochem 2014;115:1420-1429.

4. Hong SH, Ren L, Mendoza A, et al. Apoptosis resistance and PKC signaling: distinguishing features of high and low metastatic cells. Neoplasia 2012;14:249-258.

5. Lee S, Margolin K. Cytokines in cancer immunotherapy. Cancers (Basel) 2011;3:3856-3893.

6. Müller L, Aigner P, Stoiber D. Type I interferons and natural killer cell regulation in cancer. Front Immunol 2017;8:304.

7. Strauss SJ, Ng T, Mendoza-Naranjo A, et al. Understanding micrometastatic disease and anoikis resistance in Ewing family of tumors and osteosarcoma. Oncologist 2010;15:627-635.

8. Diaz-Montero CM, Wygant JN, McIntyre BW. PI3-K/Akt-mediated anoikis resistance of human osteosarcoma cells requires Src activation. Eur J Cancer 2006;42:1491-1500.

9. Wang X, Wang L, Wan M, et al. Fully phosphorothioate-modified CpG ODN with polyG motif inhibits the adhesion of B16 melanoma cells in vitro and tumorigenesis in vivo. Nucleic Acid Ther 2013;23:253-263.

10. Ren L, Khanna C. Role of ezrin in osteosarcoma metastasis. Adv Exp Med Biol 2014;804:181-201.

11. Khanna C, Wan X, Bose S, et al. The membrane-cytoskeleton linker ezrin is necessary for osteosarcoma metastasis. Nat Med 2004;10:182-186.

12. Wan X, Kim SY, Guenther LM, et al. Beta4 integrin promotes osteosarcoma metastasis and interacts with ezrin. Oncogene 2009;28:3401-3411.

13. Janeway CA Jr, Travers P, Walport M, et al. Immunobiology: the immune system in health and disease. 5th edition. New York: Garland Science; 2001. Principles of innate and adaptive immunity. Available from: https://www.ncbi.nlm.nih.gov/books/NBK27090/.

14. Keller R, Keist R, Wechsler A, et al. Mechanisms of macrophage-mediated tumor cell killing: a comparative analysis of the roles of reactive nitrogen intermediates and tumor necrosis factor. Int J Cancer 1990;46:682-686.

15. Michel T, Hentges F, Zimmer J. Consequences of the crosstalk between monocytes/macrophages and natural killer cells. Front Immunol 2013;3:403.

16. Murray PJ, Wynn TA. Protective and pathogenic functions of macrophages subsets. Nat Rev Immunol 2011;11:723-737.

CHAPTER IV

EVALUATION OF THE SAFETY AND IMMUNOBIOLOGIC ACTIVITY OF SINGLE DOSE CPG ODN 2395 IN PET DOGS WITH SPONTANEOUS OSTEOSARCOMA

Abstract

CpG ODNs are synthetic oligonucleotides composed of unmethylated CpG dinucleotides, or CpG motifs, which are recognized by TLR9 as indicative of bacterial infection, due to their much higher frequency in bacterial DNA compared to mammalian DNA. This recognition subsequently stimulates a profound inflammatory response characterized by the activation of plasmacytoid dendritic cells, release of pro-inflammatory cytokines, and ultimately activation of a T_h1-polarized T cell response. In **Chapters II** and **III** we confirmed the ability of CpG ODN 2395 to shape an anti-tumor immune response while minimizing pro-inflammatory tumorigenesis, along with identifying its potent ability to reduce metastatic OS lung tumor burden in a relevant murine model of metastatic OS. These findings encouraged us to pursue whether CpG ODN 2395 was worthy of investigation in pet dogs with spontaneous OS as a possible cancer immunotherapeutic.

Before proceeding to a full-scale clinical trial, we employed a small-scale dose-escalation study to evaluate whether a single dose of CpG ODN 2395 was safe for OS-bearing dogs and could elicit a measurable immune response with diagnostic tests easily available in a clinical oncology setting. Here we show that up to a single 2.0 mg dose of CpG ODN 2395 is tolerable in dogs with spontaneous appendicular OS and exhibits no apparent acute or long-term toxicity. Additionally, a 2.0 mg dose of CpG ODN 2395 generates measurable immunobiologic read-outs consistent with an early innate response characterized by inflammation, as measured by elevated

CRP and a probable mild inflammatory leukogram, then transitioning into an adaptive response characterized by elevated IL-2 at one-week post-treatment. These positive results form the impetus for further investigation of CpG ODN 2395 within a large-scale canine OS clinical trial that also evaluates whether CpG ODN 2395 has therapeutic efficacy in OS-bearing dogs.

Introduction

CpG ODN 2395 is a synthetic class C oligonucleotide with murine and human TLR9 activity.[1] CpG ODN's mimic bacterial DNA CpG motifs, thereby serving as the ligand for TLR9, whose main function is to recognize bacterial and viral DNA. Class C CpG's stimulate IFNα production from plasmacytoid dendritic cells (which is important for NK cell activation), induce antigen-presenting cell (APC) maturation, and directly activate B cells. Therefore, Class C CpG's are considered more immunostimulatory than either Class A (or D) or Class B (or K) CpG's which function either primarily in the induction of IFNα secretion (Class A) or B cell stimulation (Class B).[2-4] This advanced immunostimulatory capacity of CpG ODN 2395, along with its capability to stimulate TLR9 in both mice and humans, increased the likelihood of this specific CpG having activity in dogs and was a defining reason why we chose to employ CpG ODN 2395 from the beginning of the *in vitro* studies (**Chapter I**).

Based off the profound reduction of lung tumor burden and increased survival time in mice receiving the TLR9 agonist CpG ODN 2395 compared to the TLR1/2 agonist Pam3CSK4 and the TLR3 agonist Poly(I:C) (**Chapter III**), CpG ODN 2395 was determined to be a rationally justified agonist for evaluation in pet dogs with spontaneous OS. While we further elucidated in **Chapter III** that CpG ODN 2395 may have a more restricted time frame of activity in regard to preventing lung metastasis, this drug may still be clinically relevant in dogs with

spontaneous OS who do not yet have appreciable pulmonary metastatic disease. Therefore, we proceeded with a small dose-escalation pilot study to investigate the safety and immunobiologic activity of a single dose of CpG ODN 2395 in pet dogs with spontaneous OS, in an effort to assess the possible potential of this to drug to be suitable for use in a large scale clinical trial for dogs with OS. We hypothesized that single dose CpG ODN 2395 would produce a measurable immune response with minimal adverse effects.

Material and Methods

Pet Dogs with Spontaneous OS

Pet dogs with spontaneous OS were recruited through the University of Illinois College of Veterinary Medicine's Cancer Care Clinic within the Veterinary Teaching Hospital and under the guided care of veterinary medical oncologists. Dogs were eligible for the trial if they met the following criteria: 1) had a histologic or cytologic diagnosis of appendicular OS, excluding proximal femur lesions, 2) weight $\geq$ 25 kg, 3) no prior treatment with chemotherapy, radiation therapy, or bisphosphonates, and 4) no non-steroidal anti-inflammatory drugs (NSAIDS) administered in the previous 7 days prior to starting the trial. Pet owners received a monetary credit for use at the Veterinary Teaching Hospital and were gifted 3 zoledronate treatments, a bisphosphonate agent used in the treatment of OS.[5,6] Administration of CpG ODN 2395 and sample collection procedures were approved by the University of Illinois IACUC.

CpG ODN 2395 Administration and Sample Collection

Recruited dogs with spontaneous appendicular OS were administered a single injection of CpG ODN 2395 subcutaneously immediately overlying the primary tumor. Dogs received either

a 0.5, 1.0, or 2.0 mg dose of CpG ODN 2395 reconstituted in 0.5 mL of endotoxin-free water. Dose determination was based upon serial entry into the trial, in the fashion of a dose-escalation study, with a minimum of 4 dogs recruited per dose. Whole blood was collected for complete blood count (CBC), serum biochemistry, and banking of serum and plasma prior to CpG ODN 2395 treatment (day 0), 24 hours after injection (day 1), and 7 days post-injection (day 7). Urine was also collected for a complete urinalysis at day 0 and day 7. Fine-needle aspirates of the lymph node draining the primary tumor were similarly collected at all three time points, if clinically obtainable. Dogs were monitored for any adverse effects immediately following CpG ODN 2395 administration and throughout the 7-day period of the study.

Serum Cytokine Measurement

Banked serum stored at -80ºC was evaluated with canine-specific ELISA for C-reactive protein (CRP), IFNγ, IL-2, and TNFα. CRP was measured with Canine C Reactive Protein ELISA (Abcam, Cambridge, UK). IFNγ and TNFα were measured with Canine Quantikine ELISA (R&D Systems). IL-2 was measured with Canine IL-2 ELISA (MyBioSource).

Hematology, Biochemistry, and Urinalysis

All samples for CBC, serum biochemistry, and urinalysis were processed on the day of collection by standard procedures within the clinical pathology section of the University of Illinois Veterinary Diagnostic Laboratory.

Lymph Node Cytology

Lymph node samples were stained with Wright-Giemsa and evaluated by a board-certified veterinary clinical pathologist. Samples were interpreted based off changes in lymphoid populations or infiltrating inflammatory cells.

Statistics

Statistical analysis was performed with GraphPad Prism version 7.0a for Mac OS X (GraphPad Software). Significance was set at $p < 0.05$. Cytokine and hematology data were analyzed by repeated measures ANOVA and Tukey's post-test. Data is represented as the mean ± SEM. For all data, $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

Results

Recruitment of Pet Dogs with Spontaneous OS

A total of 13 dogs were recruited, which consisted of 9 spayed females and 4 castrated males. Ages ranged from 6.1 to 14.3 years with a median age of 10 years. Breeds included Alaskan malamute (1), Labrador retriever (1), golden retriever (1), Great Dane (1), Great Pyrenees (1), greyhound (2), mixed breed (5), and St. Bernard (1). Four dogs each were recruited for 0.5 and 2.0 mg treatments. Five dogs were recruited for 1.0 mg treatment. One dog (0.5 mg) did not have blood collected on day 1 and another dog (1.0 mg) did not complete the trial; these dogs were excluded from cytokine and hematology analysis. One dog (2.0 mg) was unable to have lymph node aspirates collected at any time during the trial; four other dogs (1 in the 0.5 mg group and 3 in the 1.0 mg group) also did not have lymph node aspirates collected at each time point.

Single Dose CpG ODN 2395 in Dogs with Spontaneous OS Appears Safe

No dogs experienced any adverse reactions during the 7-day study period. Additionally, no evidence of acute renal, hepatic, or GI toxicity were noted biochemically and significant life-threatening hemogram abnormalities were not observed.

High-Dose CpG ODN 2395 is Immunostimulatory in Dogs with Spontaneous OS

CRP was higher for dogs in the 2.0 mg group at day 1 compared to day 0, but was not statistically significant ($p = 0.07$). The increase in CRP normalized by day 7 in this group. No significant differences were observed for CRP in the 0.5 and 1.0 mg treatment groups (**Figure 4.1a**). Additionally, IL-2 was significantly increased at day 7 compared to day 0 in dogs receiving the 2.0 mg dose of CpG ODN 2395. No statistical difference was detected between day 1 and day 0 in this group, or at any time point for the 0.5 and 1.0 mg groups (**Figure 4.1b**). IFNγ was below the assay's limit of detection for the majority of dogs at each time point. A single dog had detectable IFNγ concentrations, but no apparent increase secondary to CpG ODN 2395 treatment (data not shown). TNFα was below the assay's limit of detection for all dogs at each time point (data not shown).

In addition to the trend towards significant CRP elevations at day 1 and the significantly elevated IL-2 at day 7 in the 2.0 mg CpG ODN 2395 group, these dogs also had significant increases in their WBC and neutrophil counts on day 1 post-treatment (**Figure 4.2b, c**). These hemogram changes could be interpreted as a cortisol-induced stress response and were not elevated outside of the reference interval, but in conjunction with the trend towards elevated CRP at this time point, these changes were suspected to represent mild inflammation. Toxic

neutrophil changes (cytoplasmic basophilia or vacuolation) were also noted in 3 dogs (75%), further supporting inflammation. Monocytes were decreased in the 0.5 mg CpG ODN at day 7 post-treatment, but was not considered a clinically relevant finding (**Figure 4.2e**). Hematocrit, lymphocytes, eosinophils, and platelets were not significantly different from baseline for each treatment group (**Figure 4.2a, d, f, g)**. Lymph node cytology also failed to identify any trends suggesting overt evidence of inflammation or antigenic stimulation in response to CpG ODN 2395 therapy within any treatment group, however, many of the lymph nodes were already reactive (increased plasma cells) prior to treatment (**Table 4.1**). Overall, these results show that a single 2.0 mg dose of CpG ODN creates measurable immunobiologic changes characterized by inflammation (elevated WBC, neutrophils, and CRP) followed by evidence of T cell activation (elevated IL-2).

Discussion

This pilot dose-escalation study reveals that single dose CpG ODN 2395 can be safely administered to dogs with spontaneous OS and that a single dose of 2.0 mg CpG ODN 2395 initiates a measurable inflammatory response characterized by initial elevations in CRP, one of the main acute phase proteins (APPs) of the dog,[7] along with elevated WBC and neutrophil counts consistent with inflammation. This acute inflammatory response is then followed by a late-term elevation in IL-2, indicative of systemic T cell activation. These results strongly suggest CpG ODN 2395 does have an immunostimulatory phenotype in OS-bearing dogs and supports further investigations into whether CpG ODN 2395 has clinical efficacy in preventing OS lung metastasis.

Although we observed increased CRP in response to 2.0 mg CpG ODN 2395, which supported acute inflammation, this change was not statistically significant. Time to peak CRP levels following an inflammatory insult in dogs have been shown to vary anywhere from 24 to 48 hours.[8,9] Thus, our day 1 sampling protocol may have missed peak CRP levels post-CpG ODN 2395 injection. Measurement of serum amyloid A (SAA), the other major canine APP,[7] may help further support our findings of acute inflammation. TNFα and IFNγ also did not increase in response to CpG ODN 2395 treatment, and in nearly all of the patients recruited were unmeasurable. The serum half-lives of most cytokines are short (often <1 hour)[10] and in combination with only a single dose of CpG ODN 2395, TNFα and IFNγ may have gone undetected due to our sampling schedule. Additionally, dogs with OS have been shown to have increased numbers of MDSCs and T_{regs}, which may be contributing to an immunosuppressive state in these patients,[11] thereby preventing production of these cytokines through the generation of alternatively-activated macrophages (M2) or inhibition of NK and T cells.[12]

We also sought to assess the safety of single dose CpG ODN 2395 in OS-bearing dogs. While the biodistribution and half-life of CpG ODN 2395 has not been reported, SQ administration of a different CpG ODN molecule in rodents at accelerated doses above those used for human clinical trials appeared safe and resulted in little long-term accumulation in organs such as the kidney.[13] Previous evaluation of a CpG ODN in tumor-bearing dogs reported minimal adverse effects[14] and we expected a single dose of CpG ODN 2395 to be safe, which was confirmed with no observed evidence of acute hepatic, renal, or GI toxicity within our study dogs, either clinically or biochemically. However, our numbers are small and additional investigations into CpG ODN 2395's safety profile in dogs should be performed if a large-scale, multi-dose study is commissioned.

While our findings support our initial hypothesis, this study's major limitation is its evaluation of only single dose CpG ODN 2395. Future studies utilizing CpG ODN 2395 in OS-bearing dogs will likely employ an injection strategy similar to those used in our murine studies (**Chapter III**), whereby dogs will receive multiple doses of CpG ODN 2395. We envision this in the setting of 1-2 doses given pre-amputation, followed by 1-2 additional doses given post-amputation, along with standard of care chemotherapy for canine OS. While these additional doses of CpG ODN 2395 may stimulate a more robust anti-tumor inflammatory response, CpG's also have the potential to induce immunosuppressive cell subsets, such as T_{regs},[15] which are already suspected to be increased in dogs with OS. Although we observed no change in T_{regs} within our murine studies with up to 6 weekly treatments of CpG ODN 2395, investigating these cellular subsets within OS-bearing dogs would be warranted and highly informative in regard to how TLR9 agonism influences long-term immune responses in cancer-bearing dogs that may already be immunosuppressed. Multi-dose CpG ODN 2395 may also be associated with toxicities not observed with single dose CpG ODN 2395; this too would also need to be evaluated and if significant long-term toxicities are noted, weighed against the benefits from any measurable positive outcomes.

FIGURES AND TABLE

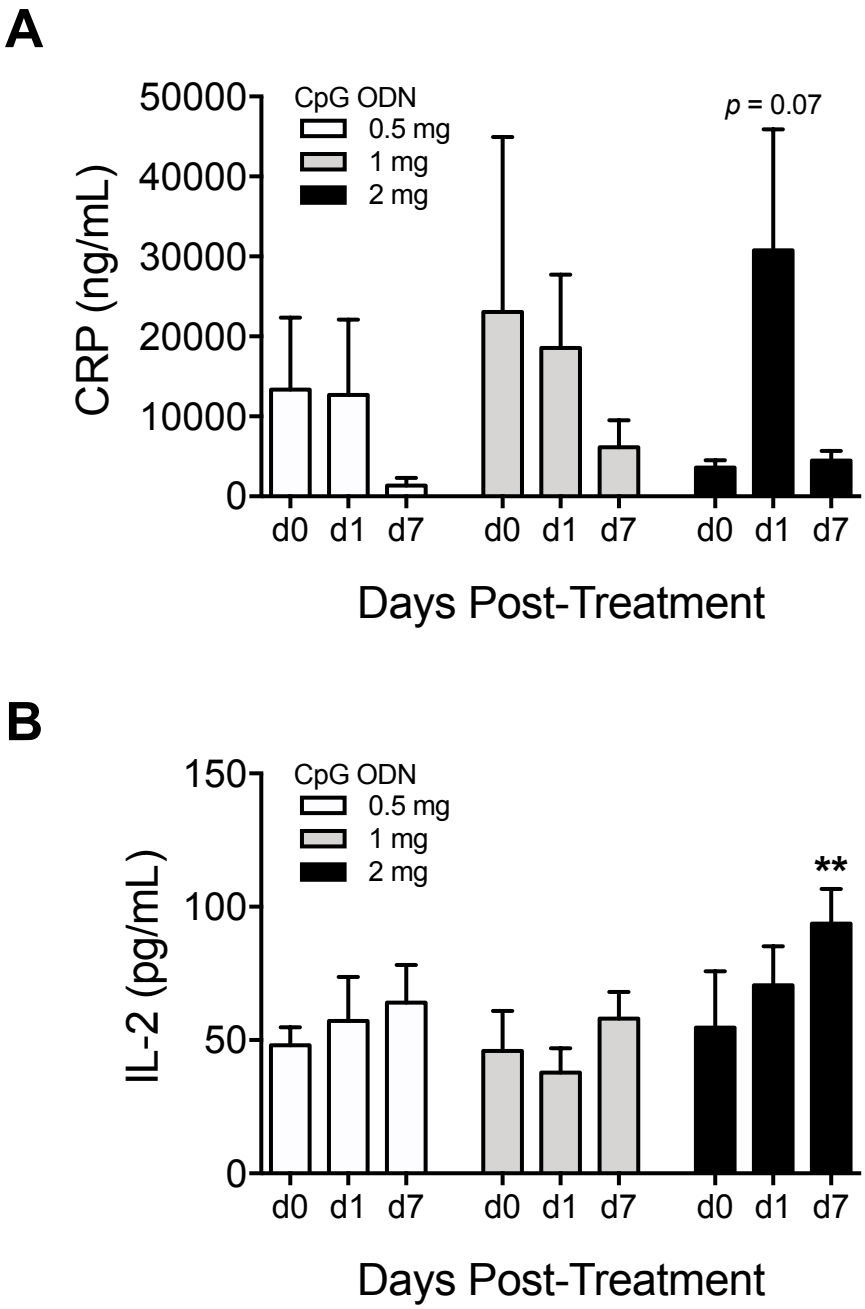

Figure 4.1: C-reactive protein (CRP) and IL-2 serum levels from pet dogs with spontaneous OS administered a single SQ injection of 0.5, 1, or 2 mg CpG ODN 2395. Serum was collected pre-treatment (d0), then at 24 hours (d1), and 7 days (d7) post-treatment. CRP levels **(A)** trend towards significance (p=0.07) 24 hours after treatment and IL-2 **(B)** is significantly increased 7 days post-treatment with a single 2 mg injection of CpG ODN 2395. Repeated measures ANOVA. **p<0.01

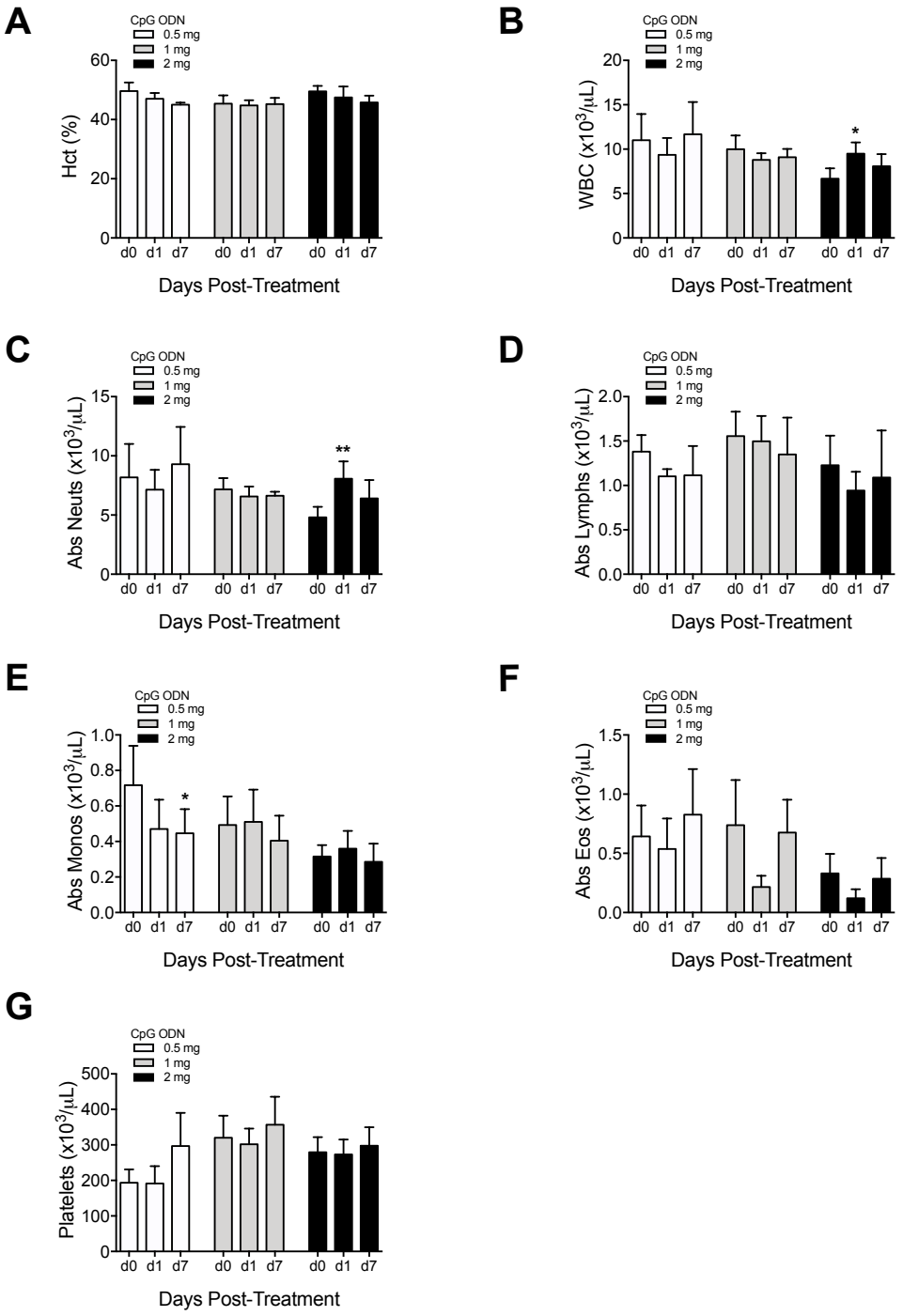

Figure 4.2: Hematology values from pet dogs with spontaneous OS administered a single SQ injection of 0.5, 1, or 2 mg CpG ODN 2395.

Blood was collected pre-treatment (d0), then at 24 hours (d1), and 7 days (d7) post-treatment. Hematocrit **(A)**, lymphocytes **(D)**, eosinophils **(F)**, and platelets **(G)** do not change regardless of CpG ODN dose. Monocytes **(E)** decreased at d7 in the 0.5 mg CpG ODN 2395 group. Total white blood cell counts **(B)** and neutrophils **(C)** are significantly increased the day after a 2 mg dose of CpG ODN 2395 (d1), consistent with an acute inflammatory response. Repeated measures ANOVA. *p<0.05 and **p<0.01

Draining Lymph Node Cytology

0.5 mg CpG ODN	Day 0	Day 1	Day 7
Dog 1	ND	NCED	NCED
Dog 2	Mildly reactive	Mildly reactive	Hemodilute
Dog 3	NCED	NCED	NCED
Dog 4	Mildly reactive	Mildly reactive	Mildly reactive
1.0 mg CpG ODN	**Day 0**	**Day 1**	**Day 7**
Dog 5	Mildly reactive	Reactive	Mildly reactive
Dog 6	Hemodilute	NCED	Reactive
Dog 7	ND	Mildly reactive	Mildly reactive
Dog 8	Mildly reactive	Mildly reactive	ND
Dog 9	NCED	NCED	ND
2.0 mg CpG ODN	**Day 0**	**Day 1**	**Day 7**
Dog 10	NCED	Hemodilute	NCED
Dog 11	Hemodilute	Hemodilute	NCED
Dog 12	Reactive + LH	Reactive	LH
Dog 13	ND	ND	ND

Table 4.1: Cytologic findings from the draining lymph node of pet dogs with spontaneous OS that were given a single SQ injection of CpG ODN 2395.
Lymph node cytology samples were taken prior to CpG ODN injection (day 0), the day after injection (day 1), and then one week after injection (day 7) to assess for evidence of immune activation. No subjective increases in reactivity or inflammation were appreciated consistently among any treatment group. LH= lymphoid hyperplasia; NCED = no cytologic evidence of disease; ND = not done, as no sample could be reasonably collected; Hemodilute = sample had excessive red blood cell contamination and minimal lymphoid population available for evaluation.

LITERATURE CITED

1. InvivoGen. ODN 2395. http://www.invivogen.com/PDF/ODN2395_TDS.pdf.

2. Häcker G, Redecke V, Häcker H. Activation of the immune system by bacterial CpG-DNA. Immunology 2002;105:245-251.

3. Martinson JA, Tenorio AR, Montoya CJ, et al. Impact of class A, B, and C CpG-oligodeoxynucleotides on in vitro activation of innate immune cells in human immunodeficiency virus-1 infected individuals. Immunology 2007;120:526-535.

4. Bauer S, Kirschning CJ, Häcker H, et al. Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. PNAS 2001;98:9237-9242.

5. Fan TM, de Lorimier LP, Garrett LD, et al. The bone biologic effects of zoledronate in healthy dogs and dogs with malignant osteolysis. J Vet Intern Med 2008;22:380-387.

6. Ohba T, Cates JM, Cole HA, et al. Pleiotropic effects of bisphosphonates on osteosarcoma. Bone 2014;63:110-120.

7. Cray C, Zaias J, Altman NH. Acute phase response in animals: a review. Comp Med 2009;59:517-526.

8. Yamashita K, Fujinaga T, Miyamoto T, et al. Canine acute phase response: relationship between serum cytokine activity and acute phase protein in dogs. J Vet Med Sci 1994;56:487-492.

9. Dabrowski R, Wawron W, Kostro K. Changes in CRP, SAA, and haptoglobin produced in response to ovariohysterectomy in healthy bitches and those with pyometra. Theriogenology 2007;67:321-327.

10. Tarrant JM. Blood cytokines as biomarkers of in vivo toxicity in preclinical safety assessment: considerations for their use. Toxicol Sci 2010;117:4-16.

11. Wycislo KL, Fan TM. The immunotherapy of canine osteosarcoma: a historical and systematic review. J Vet Intern Med 2015;29:759-769.

12. Sinha P, Clements VK, Bunt SK, et al. Cross-talk between myeloid-derived suppressor cells and macrophages subverts tumor immunity towards a type 2 response. J Immunol 2007;179:977-983.

13. Noll BO, McCluskie MJ, Sniatala T, et al. Biodistribution and metabolism of immunostimulatory oligodeoxynucleotide CpG 7909 in mouse and rat tissues following subcutaneous administration. Biochem Pharmacol 2005;69:981-991.

14. Yannelli JR, Wouda R, Masterson TJ, et al. Development of an autologous canine cancer vaccine system for resectable malignant tumors in dogs. Vet Immunol Immunopathol 2016;182:95-100.

15. Moseman EA, Liang X, Dawson AJ, et al. Human plasmacytoid dendritic cells activated by CpG oligodeoxynucleotides induce the generation of CD4+CD25+ regulatory T cells. J Immunol 2004;173:4433-4442.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

The culmination of this research has identified the TLR9 agonist CpG ODN 2395, as a highly-relevant potential immunotherapeutic for the treatment of metastatic OS in dogs. Using a top-down, integrated approach,[1] we systematically identified 3 potential TLR agonists (Pam3CSK4, Poly(I:C), and CpG ODN) for use in a well-validated murine model of metastatic OS, utilizing multiple biological read-outs to elucidate these 3 agonists as the most likely to promote anti-tumor immunity while concurrently minimizing the potential for tumorigenesis (**Chapter II**). Interestingly, even at this early stage of investigation, CpG ODN 2395 was suspected to be the least pro-tumorigenic of the 3 potential agonists, although we were cognizant these findings would not necessarily translate *in vivo*. Therefore, these 3 agonists were then employed within the K7M2 model of murine metastatic OS, investigating OS lung tumor burden, survival time, and effector cell composition and activity in response to TLR agonist therapy (**Chapter III**). The results from these *in vivo* murine studies clearly support CpG ODN 2395 as having defined and repeatable efficacy in the reduction of OS metastatic lung tumor burden and corresponding increases in survival time, along with enhancement of T_h1 and CTL lymphocyte subpopulations that are important players in anti-tumor immune responses.

We further identified that CpG ODN 2395 may have utility in a microscopic disease setting, which may have clinical relevance in dogs with spontaneous appendicular OS who present without concurrent radiographic evidence of lung metastasis. While we were unable to directly determine the mechanism(s) of action (MOA) for CpG ODN 2395 in reducing OS lung tumor metastasis, we suspect that CpG ODN's MOA functions somewhere along the metastatic

cascade between tumor embolization within the circulation and the formation of histologically apparent micrometastases within the lungs, and may not even be due to a direct cellular immune response. This theory is also supported by the lack of enhanced K7M2-specific *ex vivo* splenocyte cytotoxicity in mice receiving CpG ODN 2395 and no reversal of lung tumor burden in mice receiving a macrophage depleting agent, liposomal clodronate. While CpG ODN 2395 may not be beneficial in dogs who already have established macrometastases at the time of diagnosis, CpG ODN 2395 may be effective in dogs who have no or minimal micrometastatic disease that subsequently undergo amputation to remove the source of tumor emboli, thereby potentially preventing the formation of new micrometastases in a peri-operative limb amputation setting.

To this effect, we investigated whether CpG ODN 2395 was safe to administer in pet dogs with spontaneous appendicular OS and to determine if a single dose of CpG ODN 2395 could elicit a measurable immunobiologic response, using a dose-escalation study design (**Chapter IV**). Our results suggest up to a single dose of 2.0 mg CpG ODN 2395 is safe to administer in dogs with spontaneous OS without overt clinical, hematological, or biochemical evidence of toxicity; this dose also elicited measurable immunobiologic responses such as post-administration elevation of the acute phase protein CRP and a concurrent mild inflammatory leukocyte response, followed by subsequent elevation in IL-2, consistent with T cell activation. These findings lend further promise to employing CpG ODN 2395 successfully into a larger-scale clinical trial in OS dogs, where multi-dose safety, long-term immunobiologic activity, and survival analysis of CpG ODN 2395 would be determined. We envision this clinical trial would investigate CpG ODN 2395 in an adjuvant setting, where dogs would receive CpG ODN 2395 in a peri-operative setting (both pre- and post-amputation of the OS-bearing limb) followed by

standard of care chemotherapy. This trial encompasses one of the main future directions of this study, and we plan on submitting a proposal to obtain funding for this type of clinical trial. Ideally, if this larger clinical trial proved fruitful, CpG ODN 2395 may even have the possibility of being translated into a therapy for pediatric OS, given the many similarities between canine and human OS (**Chapter I**).[2,3]

An additional future direction also lies amongst finding the exact or a critical MOA underlying the efficacy of CpG ODN 2395. Funding has already been obtained to perform a few pilot studies investigating other potential MOA's for CpG ODN 2395, including TNFα blockade and adoptive transfer of splenocytes from tumor-bearing, CpG ODN 2395-treated, long-surviving mice into naïve mice that will be subsequently K7M2-challeneged. We also seek to identify the lowest efficacious dose of CpG ODN 2395 in our murine model of metastatic OS, as this may further inform clinical doses for dogs and reduce costs associated with CpG ODN 2395. While our research has consistently shown CpG ODN 2395 to be effective at reducing OS lung metastasis in our murine model, identifying the MOA and lowest effective dose will help inform future studies, including clinical trials, and may open the door to other potential immunotherapeutic pathways for OS in both dogs and humans.

LITERATURE CITED

1. Paolini M, Khanna C. Translation of new cancer treatments from pet dogs to humans. Nat Rev Cancer 2008;8:147-156.

2. Wycislo KL, Fan TM. The immunotherapy of canine osteosarcoma: a historical and systematic review. J Vet Intern Med 2015;29:759-769.

3. Fan TM, Khanna C. Comparative aspects of osteosarcoma pathogenesis in humans and dogs. Vet Sci 2015;2:210-230.